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The Bioactive Compounds in Agricultural Products and Their Roles in Health Promoting Functions

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THE BIOACTIVE COMPOUNDS IN AGRICULTURAL PRODUCTS AND THEIR
ROLES IN HEALTH PROMOTING FUNCTIONS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by

Yixiao Shen

B.S., Shenyang Agricultural University, 2010

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December 2015

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ABSTRACT

Bioactive compounds from different agricultural food products have attracted great interest from food industries and researchers for their health promoting functions such as antioxidant, antiaging, anti-inflammatory and anticancer performance. In this study, hydrophilic and lipophilic fraction of two economical agricultural products sweet sorghum millet and sweet potato, as well as two herbs, butterfly pea and basil were extracted. The profiles and contents of phenolics, fatty acids, tocopherols, carotenoids and phytosterols in these selected agricultural products were determined by chromatography and mass spectrum methods. Additionally, the anti-lipid-oxidation capability of sweet sorghum millet and basil, and anti-cancer potential of butterfly pea seed or petal and sweet potato were evaluated by emulsion models (cholesterol or cholesterol-linoleic acid emulsion) and cancer cell lines (HEp-2 and PC-12), respectively.

In the study of sweet sorghum millet, nine major hydrophilic phytochemicals were quantified at levels of 8.9 $\mu\text{g/g}$ for cinnamic acid to 1570.0 $\mu\text{g/g}$ for apigeninidin, and lipophilic phytochemicals including α - and γ -tocopherol, lutein and β -carotene were quantified at levels of 7.7, 145.7, 4.8, and 18.8 $\mu\text{g/g}$, respectively. The total phenolic content, scavenging DPPH activity and the ability of inhibiting cholesterol oxidation or stabilizing linoleic acid in hydrophilic extracts of the sweet sorghum millets were significantly higher than its lipophilic extracts. In Thai holy/sweet basil leaves or seeds, eight phenolics rosmarinic, caftaric, caffeic, chicoric, p-hydroxybenzoic, p-coumaric, protocatechuic acid and rutin were identified. The total phenolic content of Thai sweet basil leaves (TSBL) was significantly higher than Thai holy basil leaves (THBL), Thai holy (THBS) and sweet basil seed (TSBS). The order of scavenging DPPH free radical

activity and anti-lipid-oxidation ability from high to low was THBL, TSBL, THBS and TSBS. Butterfly pea seeds contained fifteen major phenolics such as sinapic acid, epicatechin and hydroxycinnamic acid derivative with concentrations above 0.5 mg/g FW, while its petals contained a group of ternatins (A1, B2, B3, C2, D2 and D3), flavone glycosides, delphinidin derivatives and ellagic acid. Both seeds and petals had four different phytosterols and α - and γ - tocopherol. Linoleic acid is the highest level of fatty acid in both seeds and petals, while phytanic acid was only found in the petals. The cellular study demonstrated that hydrophilic butterfly pea seed (HBS) exhibited significantly higher capability than its petal (HBP) in inhibiting the proliferation of HEP-2 cells. However, the capability of lipophilic extracts of both seed and petal were much lower than their corresponding hydrophilic extracts. In the sweet potato study, most of the phenolic compounds, fatty acids, and phytosterols significantly increased, and four more phenolic acids were found after fermentation of sweet potato due to the enzymatic action of *Lactobacillus acidophilus* LA-K compared with raw sweet potato. In the anticancer potential study, the fermented sweet potato extracts exhibited higher efficiency than raw extracts in inhibiting the cancer cell PC-12 proliferation. Also, purified hydrophilic extracts of raw or fermented extracts had greater anticancer potential than their corresponding lipophilic extracts. However, each type of extracts had little influence on the normal monkey kidney cell (CV-1) growth. Based on the dissertation research, the natural agricultural extracts could be used as health promoting ingredients in functional food or potential therapeutic ingredients for cancer treatment.

CHAPTER 1. LITERATURE REVIEW

1.1 Lipid Oxidation

Lipid oxidation involving complicated chain reactions is a major concern in food products and human health. Autoxidation, photooxidation and enzymatic oxidation are three typical oxidations occurring in food products based on the lipid substrates, oxidation agents and environmental factors (Barriuso, Astiasarán, & Ansorena, 2013). Generally, enzymatic oxidation is catalyzed by lipoxygenases, while, photooxidation is initiated by active singlet oxygen species formed by the excitation of triplet molecular oxygen under light exposure or presence of photosensitizers (Choe & Min, 2006). However, autoxidation undergoes a series of chain reactions involving free radical initiation, propagation and termination, during which several oxidized compounds such as peroxides, aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers are produced (Barriuso, Astiasarán, & Ansorena, 2013).

1.1.1 Lipid oxidation and food

Dietary lipids, either naturally existing in raw food materials or manually added during processing, have important influence on food qualities, especially the flavor and nutrition. A direct influence of lipid oxidation on high lipid food is the production of flavors and odors (Eskin et al., 2013). Although lipids play an important role in contributing to the special aroma characteristics of cooked food, they easily undergo oxidation and deterioration during processing or storage (Wsowicz et al., 2013). Primary flavor compounds can be lost, while secondary compounds can be formed and usually cause rancidity, which is known as characteristic of a variety of pungent, and oily off-odors.

The primary off-flavor compounds, particularly aldehydes and ketones were listed in Table 1.1 (Eskin et al., 2013).

Table 1.1 Off-flavors produced by various lipid oxidation products

Compound ^a	Off-Flavor	Threshold Value (ppm)		
		In Oil		In Water
		Odor	Taste	Odor
Aldehydes				
Pentanal	Sharp, bitter almond	0.24	0.15	0.012
Hexanal	Green—fruity, bitter almond	0.32	0.08	0.008
Octanal	Fatty, soapy—fruity	0.32	0.04	0.0007
Nonanal	Tallowy, soapy—fruity	13.5	0.2	0.001
Decanal	Orange peels	6.7	0.7	0.0001
Nonenal (3c)	Green cucumber	0.25	0.03	
Nonenal (2t)	Tallowy, starch—glue	3.5	0.04	0.0008
Nonadienal (2t,4t)	Fatty, oily	2.5	0.46	
Nonadienal (2t,6c)	Cucumbers	0.01	0.0015	
Nonadienal (2t,6t)	Tallowy, green	0.21	0.018	
Decadienal (2t,4c)	Frying odor		0.02	
Decadienal (2t,4t)	Deep-fried	2.15	0.1	
Ketones and furans				
1-Pentene-3-one	Sharp, fishy		0.003	
1-Octen-3-one	Metallic			
1-Octen-3-ol	Moldy, mushroomy	0.077	0.0001	0.00009
2-Pentylfuran	Buttery, beany	2		
2-(1-Pentenyl)furan (<i>cis</i> and <i>trans</i>)	Licorice	2 to 6		

Source: Eskin et al. (2013)

For example, both adipose tissue and intramuscular fat contents in meat are prone to get oxidized and degraded under constant exposure to lights, high temperature, metal or other sensors to create a prolific number of volatile or off-flavor compounds (Akyar, 2012). Due to their low odor threshold, the presence of lipid oxidation degraded products; even at relatively low concentration, it could impair the food sensory properties (Akyar, 2012). In the study of Grosh et al (1992), compared with fresh sample, eight times higher levels of the unpleasant fatty off-flavor including vinyl ketone, 1,5-octadien- 3-one and 2-

nonenal, were generated in butter fat after 42 days of storage. In addition, the increase in the concentration of carbonyls such as 2-nonenal in butter oil or 1,5-octadien-3-ol in boiled frozen trout significantly increased, replacing their original aroma with fatty, tallowy, green off-odors (Grosch et al., 1994). In fried food or aging oil, a group of volatile compounds including pentane, 2-heptenal, isomers of 2,4-heptadienal, and isomers of 2,4-decadienal occur. With an increasing storage time, the concentration of aldehydes and ketones pyridines, sulfides, thiazoles, alcohols, phenols and esters in fried food or oil were several times higher than the original level (Min, & Schweizer, 1983). Moreover, trimethylamine, as well as oxidized polyunsaturated fatty acids are responsible for the off-flavors of products containing fish oil (Van Ba, Touseef, Jeong, & Hwang, 2012). Generally, lipid oxidation not only generates off-flavors of food, but also gives rise to the loss of essential amino acids, fat-soluble vitamins, and other bioactive compounds.

1.1.2 Lipid oxidation and health

In human body, free radicals could be generated internally in the normal metabolism or by reactive oxygen species (ROS) in some external condition such as smoking, pollution, and radiation. Especially, ROS induces the production of lipid peroxidation products such as peroxides and aldehydes which then diffuse from their site of generation and inflict damage at remote locations (Ramana, Srivastava, & Singhal, 2013). Therefore, initiated by ROS, oxidized lipid products are able to propagate the responses and cause dysfunction in biological tissue or organ injury (Ramana, Srivastava, & Singhal, 2013).

Generally, the oxidation of fatty acids is one of the most fundamental reactions in lipid chemistry. Most oxidative degradation of fatty acids occurs in cellular membranes

such as mitochondria, microsomes, peroxisomes and plasma membrane (Boveris, & Navarro, 2008). The onset of lipid peroxidation within biological membranes is associated with changes in their physicochemical properties and with alteration of biological function of lipids and proteins (Repetto et al., 2012). As a result, the toxicity of lipid peroxidation products will further involve in neurotoxicity, hepatotoxicity and nephrotoxicity (Boveris et al., 2008). Several studies have demonstrated that liver, kidney, and heart are major target organs for oxidative damage (Eder, 1999). Oxidative damage in liver is associated with hepatic lipid metabolism, and symptoms of the change of liver weights and hepatic bile duct lesions were observed in test animals by administration of oxidized oils and fats in the study of Eder (1999). Similar results showed that lipid oxidation induced morphological damages and mitochondrial swelling would further affect the organ structure and malfunction of the bile canaliculi as well as the absorption and transportation mechanisms of α -tocopherol (Repetto et al., 2012). Also, cardiac fibrotic lesions and necrosis were observed in heart due to the inflammatory response from lipid peroxidation and disorder (Repetto et al., 2010). It has been reported that fatty acid peroxides could accelerate three key phases of atherosclerosis which includes endothelial injury in initiation, accumulation of plaque in progression and thrombosis in termination (Singh et al., 2002). With the stimulation of endothelium, inflammatory response starts to display adhesive molecules for circulating white blood cells and producing cell type-specific agonists (McIntyre & Hazen, 2010). The vascular endothelial cells will generate stimuli or plague by the activation of those agonists that are associated with atherosclerosis, heart attack, Alzheimer's disease, rheumatic arthritis and other chronic diseases (McIntyre & Hazen, 2010).

In addition, there was a strong relationship between lipid peroxides and DNA damage or development of cancer in humans (De Bont & van Larebeke, 2004). The epoxy aldehydes from lipid oxidation are reactive in cross-linking reactions with proteins and easily attack electron-rich centres in DNA, generating chemically altered bases known as DNA adducts (Luczaj & Skrzydlewska, 2003). A group of lipid oxidation derived compounds such as propeno and substituted propano adducts of deoxyguanosine with malondialdehyde (MDA), acrolein, crotonaldehyde and etheno adducts, leads to promutagenic lesions, responsible for the mutagenic and carcinogenic effects (Luczaj, & Skrzydlewska, 2003). The lipid oxidized products will further alter protein properties by the reaction with lysine amino, cysteine sulfhydryl, and histidine imidazole groups (Esterbauer, 1996). Thus, the modifications of protein result in neurodegenerative disorders, kinases activation and nuclear transcription inhibition (Uchida, 2003; Camandola et al., 2000).

1.2 Antioxidant

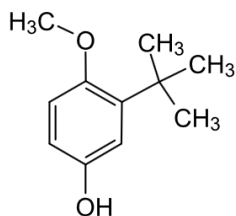
Naturally, there is a dynamic balance between pro-oxidants and antioxidants to allow the body to maintain the normal physiological conditions (Rahal et al., 2014). The interference of the balance in any direction in the redox potential will lead to deleterious results for organelle and biological site (Repetto et al., 2012). The oxidative damage that occurs by an increase in the pro-oxidant over the capacity of the antioxidant is defined as oxidative stress; while the reductive stress is a result of an increase in the reducing power (Repetto et al., 2012). However, the protective antioxidant is only available to deal with physiological rate of free-radical generation. In other words, the external and excessive free radicals which generated from living environment that cannot be eliminated in time

and damage biologically relevant molecules such as proteins, lipid or carbohydrates (Ashok & Sushil, 2005). Therefore, the antioxidant molecule or enzymatic systems play important roles against, retard or avoid undesired oxidations in the body defense system (Vivek & Surendra, 2006).

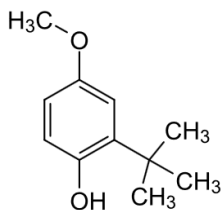
1.2.1 Categories of antioxidants

1.2.1.1 Synthetic antioxidants

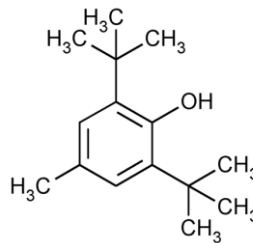
Owing to the low cost and efficient performance, synthetic antioxidants are commonly used during the storage and distribution of food products. Based on their action mode, the synthetic antioxidants could be defined as primary antioxidants, which play important roles in breaking the chain reaction of oxidation by hydrogen donation and generating more stable radicals; while the secondary antioxidants which slow the oxidation rate by metal chelation, primary antioxidant regeneration, decomposition of hydroperoxides, singlet oxygen deactivation, ultraviolet radiation absorption, and oxygen scavenging (Flora, 2009; Barbut, 2010). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG), dodecyl gallate (DG) and octyl gallate (OG) are a group of free radical terminators (Figure 1.1).

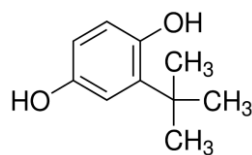


Butylated hydroxyanisole (BHA)

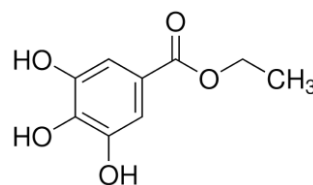


Butylated hydroxytoluene (BHT)

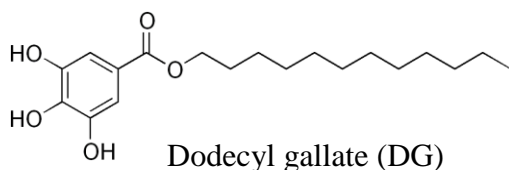




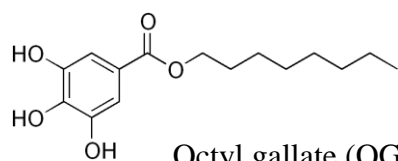
tert-Butylhydroquinone (TBHQ)



Propyl gallate (PG)



Dodecyl gallate (DG)



Octyl gallate (OG)

Figure 1.1 Chemical structures of the synthetic antioxidants

Among them, BHA is most effective in preserving food color and flavor as well as animal fats with short chain fatty acids during baking and frying (Devlieghere, Vermeiren, & Debevere, 2004). The combination use of BHA and BHT are shown to have synergistic effects, especially in nut products (Shahidi et al., 1992). TBHQ is an effective supplement in color improvement and helps to increase oxidative stability in the process of liquid oil hydrogenation and fried food (Shahidi & Zhong, 2005). Along with citric acid, PG is commonly used in stabilizing vegetable oil or animal fats and chewing gum bases (Shahidi & Zhong, 2005). Octyl gallate (OG) and dodecyl gallate (DG) are the other two gallate antioxidants used as food additives in shortening, baked goods, candy, chewing gum and dried milk (van der Heijden, Janssen, & Strik, 1986). As for the chelating agent, EDTA could form complexes with pro-oxidative metal ions and is mostly used in processed fruits and vegetables, salad dressings, soft drinks, margarine and canned fish (Rangan & Barceloux, 2009).

However, an excessive dose of synthetic antioxidants implicates carcinogenicity, liver lesion, haemorrhaging, etc., which are harmful to human health, thus, they are

strictly limited in food applications (Augustyniak et al., 2010). Even at a normal level but long term of ingestion, the synthetic antioxidant would assist in modifying acute toxicity of carcinogenic and mutagenic effect depending on their pattern of metabolism (Ames, Profet, & Gold, 1990). For example, BHA has been evidenced to cause hypertrophy of liver, thyroid, adrenals and lungs and cell lysis or alteration of lipid composition in serum and platelets (Gould, 1995). On the other hand, BHT could inhibit DNA repair in human lymphocytes and cause introduction of chromosomal and sperm abnormalities along with the interference with leukemia cell differentiation (Chun et al., 2006). In addition, a morphological observation showed cell loss and the induction of cell death by necrosis and apoptosis with the treatment of certain amount of PG (Zurita et al., 2007). Therefore, each synthetic antioxidant has a limitation for consumption and the potential common sources are also listed in Table 1.2.

Table 1.2 Morphology, and limits of synthetic antioxidants used in foods

Name	Morphology	Limit in food	Found in	Banned in
BHA	White waxy flakes	<200mg/kg	Cereal, chewing gum, potato chip, vegetable oil	Japan
BHT	White crystalline powder	<100mg/kg	Honey, cakes, meat products	None
PG	White crystalline powder	<200mg/kg	Spices, sugar, milk product	None
TBHQ	Beige colored powder	Not allowed	Cheese, honey, sea food	Canada, Japan, European countries
OG	White to creamy white crystalline solid	<200mg/kg	Oil, meat product, cereals, honey,	None
DG	White to creamy white crystalline solid	<200mg/kg	Dairy and meat products	None
EDTA	White crystalline powder		Salad dressing, soft drink, mayonnaise	None

Source: Goodman (1980); Morton et al. (2000)

1.2.1.2 Natural antioxidants

Different from synthetic antioxidants, most of the natural antioxidants originate from plant sources such as fruits and vegetables which are much safer than the synthetic ones (Tayel & El-Tras, 2012). Commonly, vitamins, carotenoids, phenolic acids, and flavonoids are the primary four types of natural antioxidants.

As one of the most important vitamins, ascorbic acid (vitamin C) is a water soluble antioxidant and particularly abundant in citrus fruits (Marti, Mena, Canovas, Micol, & Saura, 2009). It has been proved that the absorption of dietary vitamin C could be up to 50% at a relatively lower dose of intake (< 39 mg) (Levine et al., 1996). The participation of vitamin C in numerous oxidation-reduction reactions involves synthesis of connective tissue of sulfate and collagen and improvement of iron adsorption in gastrointestinal (GI) tract (Houglum, Brenner, & Chojkier, 1991). Deficiency of vitamin C results in chronic alcoholism, scurvy, inflammatory bleeding gums, loss of teeth, arrested skeletal development, dry skin, joint pain and increased susceptibility to the infections (Hacisevkd, 2009). Vitamin E, a group of tocopherols, is a lipophilic antioxidant which is abundant in cereal grains, nuts, spinach, oliver or flaxseed oil (Urquiaga, & Leighton, 2000). It encompasses four types of four tocopherols (α , β , γ , δ) and four tocotrienols (α , β , γ , δ), among which, α -tocopherol has the biggest poportion in nature source and the highest biological activity based on fetal resorption assays (Figure 1.2) (Weiser, Riss, and Kormann, 1996). In general, the availability of vitamin E is relatively low which is about 20-40% of the dietary intake; however, its bioavailability could be enhanced by taking it in conjunction with dietay fats (Daniel, 1986). The

symptoms of vitamin E deficiency appear as neuromuscular abnormalities characterized by spinocerebellar ataxia and myopathies (Brigelius-Flohé & Traber, 1999).

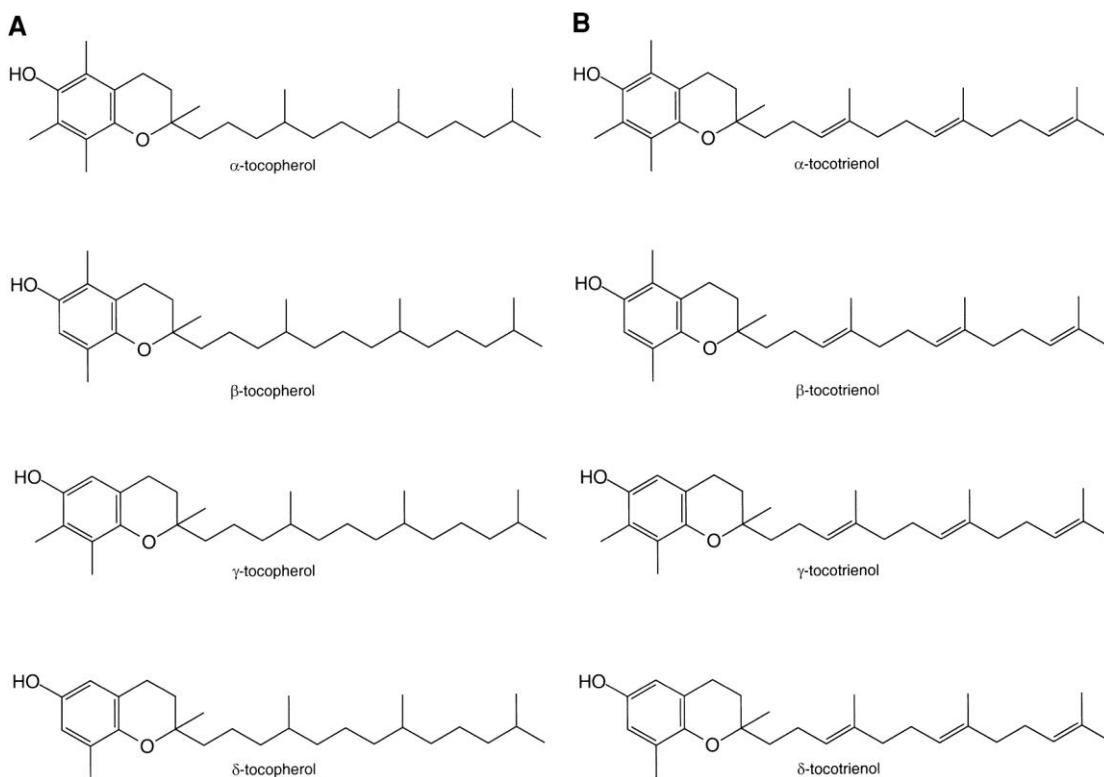


Figure 1.2 The chemical structures of four tocopherols and tocotrienals Source: (Brigelius-Flohé & Traber, 1999)

Carotenoids are a group of natural pigments of the polyene type, consisting of a ubiquitous group of isoprenoid (Figure 1.3) (Fiedor & Burda, 2014). As lipophilic molecules, β -carotene and lycopene with strict hydrocarbons in their structures are located within the inner section of the lipid bilayer, while lutein and zeaxanthin with attached oxygen atoms are arranged roughly perpendicular to the membrane surface (Wiśniewska, & Subczyński, 1998; Wiśniewska, & Subczyński, 2006). Also, β and α -carotene and β -cryptoxanthin perform as provitamin A precursors which have the capacity to be converted to vitamin A, helping growth and reproductive efficiency, maintenance of epithelial tissues and immune response (Scott & Rodriguez-Amaya,

2000). The major dietary sources of carotenoids include pumpkins, sweet potatoes, oranges, tomatoes, mangoes, papayas and yellow or red bell peppers, etc. (Xu, 2012). The intestinal absorption of carotenoids undergoes initially incorporation into mixed lipid micelles in the lumen, then, followed by the uptake into intestinal mucosa (Deming, & Erdman, 1999). After incorporation into chylomicrons, the carotenoids will be released into the lymph (Harrison, 2010). It has been reported that only about 5% of the carotenoids could be absorbed by the intestine, whereas more than 50% are from micellar solution (Olson, 1994). However, the deficiency of carotenoids has the consequences of xerophthalmia, blindness and premature death (Sommer, 2008).

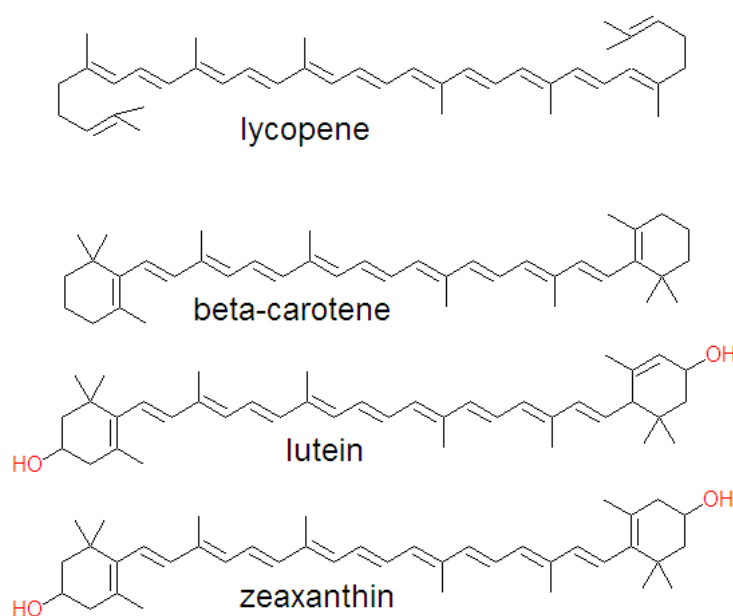


Figure 1.3 The chemical structures of carotenoids

Source: Fiedor & Burda (2014)

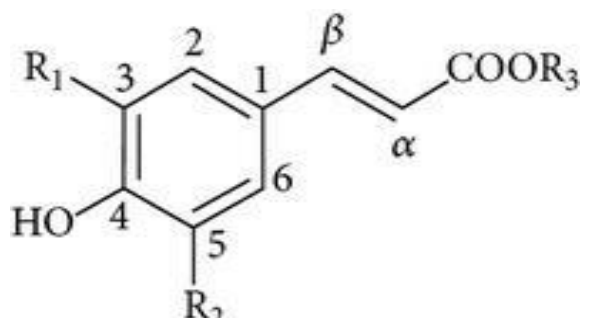
Phenolic acids are referred to the aromatic secondary plant metabolites, possessing one carboxylic acid group; they are found in fruits, vegetables and product derivatives (Saxena et al., 2012). Generally, they are subdivided into two major groups:

hydroxybenzoic acids and hydroxycinnamic acids (Manach, Scalbert, Morand, Rémésy & Jiménez, 2004). Hydroxycinnamic acid is a simple ester with glucose or hydroxy carboxylic acid, consisting of a phenylpropanoid C6-C3 structure, and considered to be the major subgroup of phenolic acids (Table 1.3) (Teixeira et al., 2013). Hydroxycinnamic acids mainly consist of *p*-coumaric, caffeic, ferulic, and sinapic acids etc., and are important in the biosynthesis of complicated phenolic systems in food and beverage such as tea leaves, coffee, red wine, and various fruits, vegetables and whole grains (Teixeira et al., 2013). Hydroxycinnamic acids exist either in free form or in conjugated forms, including mono- or polyamines, amino acids, peptides and esters as well as glycosides (Teixeira et al., 2013). The variety of hydroxycinnamic acid in plants depends on the species of the plants; however, their levels are up to 75% of the total phenolic acids (Terry, 2011). Also, it was found that derivatives of cinnamic acid are much higher in the outer parts of ripen fruits than in other parts (Ribera et al., 2010). The association of hydroxycinnamic acids with the reduced risks of cardiovascular disease, cancer and other chronic diseases could be explained by the ability to scavenge free radicals and pro-oxidant metals ability as well as modulation of the specific enzymes activity and inhibition of cell proliferation (Spencer et al., 2008; Manach, 2004). The performance of general antioxidant capability of the hydroxycinnamic acids in some lipid peroxidation systems can be expressed by the inhibition of malondialdehyde formation (Laranjinha et al., 1994).

On the other hand, the hydroxybenzoic acids are a group of tannins and lignins which with either hydroxylations or methoxylations of the aromatic ring (Manach et al.,

2004). The benzoic acid derivatives stem from the side chain degradation by loss of an acetate of the corresponding hydroxycinnamic acid derivatives, or from an intermediate

Table 1.3 Hydroxycinnamic acids and ester derivatives.

			
	R ₁	R ₂	R ₃
p-Coumaric	H	H	H
Caffeic acid	OH	H	H
5-Bromocaffeic acid	OH	Br	H
Methyl caffeate	OH	H	CH ₃
Ethyl caffeate	OH	H	CH ₂ CH ₃
Ethyl 5-bromocaffeate	OH	Br	CH ₂ CH ₃
Propyl caffeate	OH	H	CH ₂ CH ₂ CH ₃
Butyl caffeate	OH	H	CH ₂ (CH ₂) ₂ CH ₃
Hexyl caffeate	OH	H	CH ₂ (CH ₂) ₄ CH ₃
3,4,5-Trihydroxycinnamic acid	OH	OH	H
Ethyl 3,4,5-trihydroxycinnamate	OH	OH	CH ₂ CH ₃
Ferulic acid	OCH ₃	H	H
5-Bromoferulic acid	OCH ₃	Br	H
Methyl ferulate	OCH ₃	H	CH ₃
Ethyl ferulate	OCH ₃	H	CH ₂ CH ₃
Ethyl 5-bromoferulate	OCH ₃	Br	CH ₂ CH ₃
Propyl ferulate	OCH ₃	H	CH ₂ CH ₂ CH ₃
Butyl ferulate	OCH ₃	H	CH ₂ (CH ₂) ₂ CH ₃
Hexyl ferulate	OCH ₃	H	CH ₂ (CH ₂) ₄ CH ₃
Sinapic acid	OCH ₃	OCH ₃	H
Methyl sinapate	OCH ₃	OCH ₃	CH ₃
Ethyl sinapate	OCH ₃	OCH ₃	CH ₂ CH ₃
Propyl sinapate	OCH ₃	OCH ₃	CH ₂ CH ₂ CH ₃
Butyl sinapate	OCH ₃	OCH ₃	CH ₂ (CH ₂) ₂ CH ₃

in the shikimate pathway, involving in a series of enzymatic reactions (Herrmann, 1995). Its derivatives mainly refer to p-hydroxybenzoic, protocatechuic, salicylic, vanillic, syringic and gallic acids (Figure 1.4) (Rocha, Monteiro, & Teodoro, 2012). Gallic acid may be conjugated as its dimer, and trimer, and are able to be esterified to glucose in hydrolysable, condensed, or derived tannins and quinic acid (Clifford, & Scalbert, 2000). Protocatechuic acid is considered to be a therapeutic compound which generates potential antioxidant, antiulcer, antiageing, antihyperlipidemic, antifibrotic and anti-inflammatory activities (Kakkar & Bais, 2014). The biological properties of p-hydroxybenzoic include antiviral, antimutagenic, and anti-inflammatory activities, and it has been used as hypoglycemic or terarogenic agent (Manuja et al., 2013).

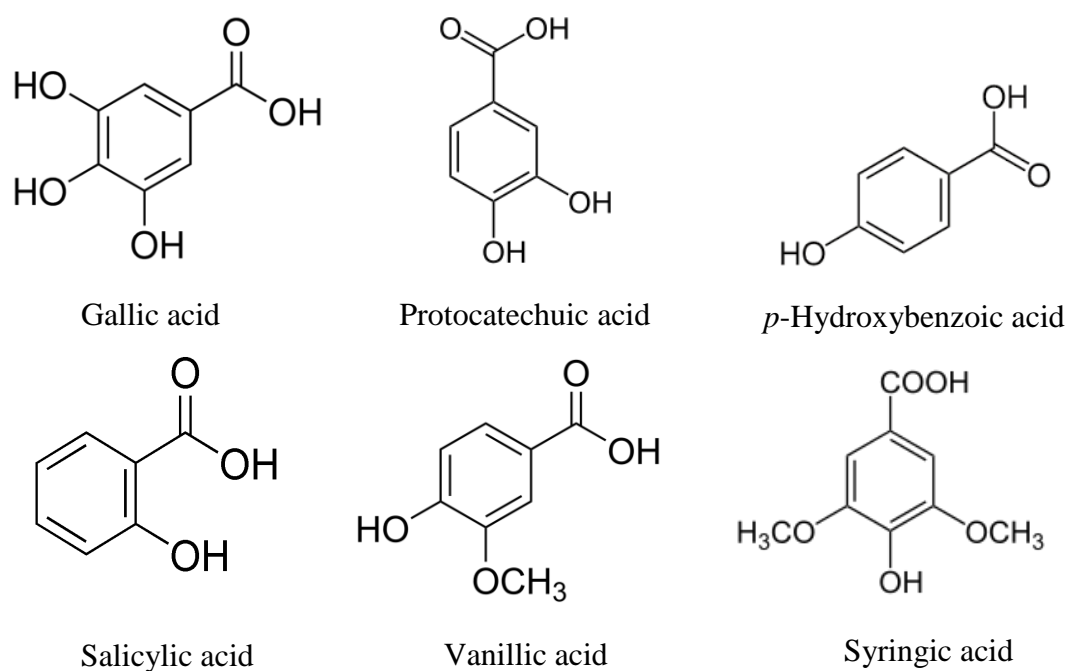


Figure 1.4 The chemical structure of hydroxybenzoic acids

Source: Khadem & Marles (2010)

Flavonoids are a special class of phenolics in which flavonoid groups have low toxicity in mammals and are widely distributed in plant kingdom (Manach et al., 2004). All flavonoids have the common C6-C3-C6 structural skeleton, consisting of two aromatic rings (A and B) linked through a heterocyclic pyrane ring (C) with one oxygen atom (Figure 1.5).

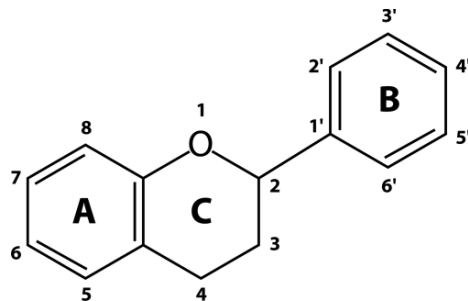
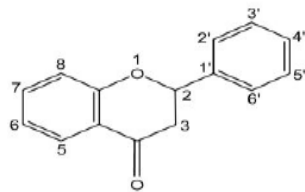


Figure 1.5 The basic structure of flavonoid

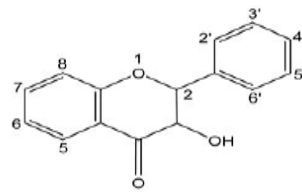
Primary dietary sources of flavonoides could be subdivided into six subclasses in the form of flavonols, flavones, isoflavones, flavonones, flavan-3-ol and flavanol which could be easily found in a variety of fruits and vegetables such as berries, soybeans and purple onion and cabbage (Figure 1.6) (Beecher, 2003).

Flavones



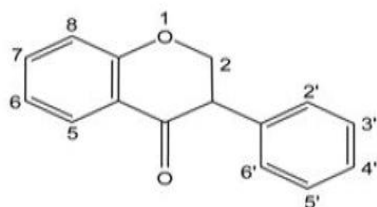
Position	5	7	3'	4'
Apigenin	OH	OH	—	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	—	—

Flavonols



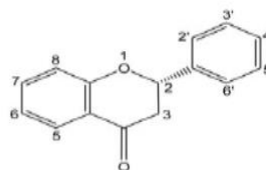
Position	5	7	3'	4'	5'
Quercetin	OH	OH	OH	OH	—
Kaempferol	OH	OH	—	OH	—
Galangin	OH	OH	—	—	—

Isoflavones



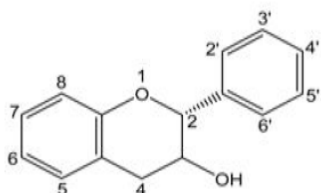
Position	5	7	4'
Ganistein	OH	OH	OH
Daidzein	—	OH	OH

Flavanone



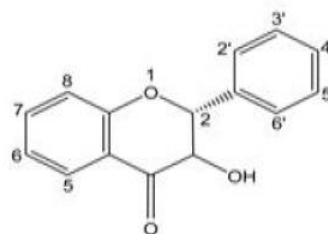
Position	5	7	3'	4'
Naringenin	OH	OH	—	OH
Hesperetin	OH	OH	OH	OCH ₃

Flavan-3-ol



Position	3	5	7	3'	4'	5'
(+)-Catechin	βOH	OH	OH	OH	OH	—
(-)-Epicatechin	αOH	OH	OH	OH	OH	—
(-)-Epigallocatechin	αOH	OH	OH	OH	OH	OH

Flanol



Position	5	7	3'	4'
Taxifolin	OH	OH	OH	OH

Figure 1.6 Different classes of flavonoids and their substitution patterns

Source: Beecher (2003)

The role of flavonoids is not only to provide attractive colors to plant but also to help promote physiological survival by regulating hormones, morphogenesis and photosynthesis as well as preventing from fungal pathogens and UV-radiation (Harborne & Williams, 2000). Recently, many studies have shown that flavonoids maintain broad biological and pharmacological effect on blood vessels, platelets, and lipoproteins and may exhibit an excellent performance of reducing the risks of coronary heart disease by modulating cardiac antioxidant defenses (Mazza, 2007). Moreover, the digestion of flavonoids assists improvement of antioxidant status and displays cholesterol-lowering

and distinct hypoglycemic actions (Prior, & Wu, 2012). In addition, flavanoids has the potential to ameliorate hyperglycemia and enhance insulin sensitivity via activation of AMP-activated protein kinase (AMPK) (Taikanwa et al., 2010)

1.3 Antioxidant Rich Food

Generally, agricultural products are recognized as important sources of a wide array of phytochemicals and dietary micronutrients (Yahia, 2010). They are able to generate favorable effects on the control of various chronic diseases and play a protective role in health maintenance (Stea et al., 2008). Obtained from diet, antioxidants could neutralize excessive ROS produced during physiological processes such as increased physical activity, tissue ischemia, reperfusion, inflammation, and mental stress or depression (Singh, Shashwat, & Suman, 2004). Therefore, the intake of antioxidant-rich food is necessary to improve health and quality of life.

1.3.1 Fruits, vegetables and herbs

Berry fruits (blackberries, raspberries, blueberries, cranberries, bilberries, strawberries, crowberries, cloudberry etc.) with purple, black or red color are abundant in anthocyanins and flavan-3-ols hydroxybenzoic or hydroxycinnamic acid derivatives, condensed and hydrolyzable tannins, etc. (Howard & Hager, 2007). Those bioactive compounds have been evidenced to contribute to antioxidant capability in a LDL (Low-density lipoprotein) oxidation model (Heinonen et al., 1998). Also, in the study of Tsuda et al. (2003), induced by the high-fat diet, the mice fed up with cyaniding-3-O-glucoside have lower body weight gain and white and brown adipose tissue weights compared with control groups. Grapes maintained a significant quantities of anthocyanins, catechin, gallic acid, and resveratrol and their levels are correlated with anti-lipid-oxidation

capabilities in human body (Frankel et al., 1998). Catechin, procyanidins, flavonols, hydroxycinnamic acid and dihydrochalcones are primary phytochemicals ranging from 0.5-2.7% on dry weight basis in apples (Xu, 2012). In an animal study, the extract of apple flesh and peels were reported to prevent macro- and microscopic damage and barrier dysfunction along the gastrointestinal tract (Carrasco-Pozo et al., 2011). Color peppers commercial available in markets contain carotenoids, vitamin A, C and E, ferulic acid, sinapic acid, quercetin, luteolins and apigenins (Frank et al., 2001). The presence of those bioactive compounds enables the color peppers to have capability of preventing free radical oxidation, cardiovascular disease, cancer and neurological disorders (Xu, 2012). Similarly, the culinary herbs such as basil, ginger, thyme and rosemary are used to enhance and complement the flavors of various foods (Xu, 2012). Having various kinds of phenolic acids, including carnosic acid, carnosol, rosmarinic acid, curcumin, eugenol etc., the culinary herbs showed strong antioxidant potential and medicinal benefits (Shan et al., 2005).

1.3.2 Cereals

Different from fruits and vegetables, vitamin E (mainly tocopherols and toctrienols) is the most abundant antioxidant and within the germ of whole-grain cereals (Souci et al., 2000). For example, rice, containing α - and β -tocopherol, α - and β -tocotrienol, and γ -orazanol are demonstrated to reduce serum cholesterol level, the risk of tumor formation and inflammatory action (Tsuji et al., 2003). Similar to other cereal grains, oats have relatively high amount of vitamin E, while it is also a great source of phenolic acids such as vanillic and *p*-hydroxybenzoic acid (Peterson, 2001). The lipophilic extract of oats could effectively inhibit oxidation of unsaturated long chain fatty acids and prevent

production of toxic cholesterol oxidized products, and is used as the natural food preservatives (Sun et al., 2006). Additionally, corn is confirmed as a common raw material for producing edible oil, in which, the concentration of vitamin E could be up to 900 mg/kg (Xu, 2012). Apart from tocopherols and phenolic acids, corn is a rich source of carotenoids such as α - and β -carotene, β -cryptoxanthin, and zeaxanthin, having a major function to prevent age-related macular degeneration (AMD) and cataracts and cell mutation (Johnson, 2000). Soybean is another cereal family in possess of various types of isoflavones with β -glucoside, daidzin, glycitin and genistin (Xu, 2012). They have been reported to show the capability of retarding the progression of Alzheimer disease and osteoporosis, and preventing harmful proliferation of cells (Zhao, et al., 2002).

1.3.3 Root vegetables

As the most common root vegetables, potatoes and sweet potatoes are two staple crops in tropical and subtropical areas (Mark et al., 2009). Currently, it occupies a dominant place in the agricultural production of Asia and Africa countries. The nutritional values of sweet potato are listed in Table 1.4. Specially, carbohydrates of sweet potato could provide energy in the human diet with a lower intake of lipids (Oke, & Workneh, 2013). The dietary fiber is believed to reduce the incidence of colon cancer, diabetes, heart diseases and digestive disturbances (Vimala, Nambisan, & Hariprakash, 2011). The flesh color of the sweet potato varies from yellow to dark-orange depending upon the levels and types of carotenoids (Vimala, Nambisan, & Hariprakash, 2011).

Carotenoids are reported to be responsible for the enhancement of immune system and inhibition of age-related macular degeneration and cataract formation (Byers & Perry 1992). Also, the purple sweet potato becomes more and more popular due to the unique

Table 1.4 Sweet potato chemical composition

Nutrient	Unit	Composition
Calories	kJ/s	130
Calories from fat	g	0.39
Protein	g	2.15
Carbohydrate	g	31.56
Dietary Fiber	g	3.9
Sodium	mg	16.9
Potassium	mg	265.2
Calcium	mg	28.6
Folate	mcg	18.2
Vitamin C	mg	3.1 (excellent source)
Vitamin A	IU	18443 (excellent source)

Source: USDA, 2009.

and abundant anthocyanins which have been reported to suppress the development of atherosclerotic lesions and oxidative stress in an *in vivo* study (Miyazaki et al., 2008). Furthermore, both sweet potatoes and potatoes contain diverse phenolics such as chlorogenic, gallic, protocatechuic and caffeic acid as well as quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside and (+)-catechin (Xu, 2012). Lotus root is an herbaceous, perennial sacred aquatic plant belonging to Nelumbonaceae family (Du et al., 2010). It is well known from its medical functions such as the treatment of all manners of bleeding and hematemesis, anti-inflammatory, antipyretic and antianxiety properties and hepatic protection (Mukherjee et al., 2009). Although carrots and radish are another commonly consumed root vegetables, their phytochemical profile and antioxidant activity are relatively lowered than other roots.

1.4 Evaluation of Antioxidants Activity

1.4.1 *In vitro* methods

1.4.1.1 Spectrophotometric assay

The antioxidant compounds have been well extracted, determined and quantified. Recently, their antioxidant potential and effectiveness have raised scientists and

consumers' concern. As a result, various *in vitro* assessments of antioxidant activities have been developed (Table 1.5). Based on the mechanisms, the antioxidant evaluation methods could be generally catalogued into hydrogen atom transfer methods (HAT) and electron transfer methods (ET) (Badarinath et al., 2010). HAT is a ubiquitous reaction involves the transfer of proton and electron for further measuring the free radical quenching by hydrogen donation and analyzing the destructive effects of reactive oxygen species (Mayer, 2011). The ET method aims at determining the ability to transfer one electron to reduce any compounds such as metals, carbonyls, and radicals (Prior, Wu, & Schaich, 2005).

DPPH free radical scavenging activity The molecule 1, 1-diphenyl-2-picrylhydrazyl (a,a-diphenyl-bpicrylhydrazyl; DPPH) is characterized as a relatively stable free radical with deep violet color by virtue of the delocalization of the spare electron over the molecule which is not associated with a single atom or covalent bond (Alam, Bristi, & Rafiquzzaman, 2013). The change in optical density of the DPPH free radical caused by the donation of hydrogen atom from a potential antioxidant could be determined under the wavelength of 517 nm (Alam, Bristi, & Rafiquzzaman, 2013).

2,2'-Azinobis-(3-Ethyl-Benzothiazoline-6-Sulphonic Acid)/ Trolox equivalent antioxidant capacity (ABTS/TEAC) In the ABTS assay, ABTS is incubated with a peroxidase and a relatively stable radical cation, and ABTS⁺ is formed, producing a relatively stable blue-green color, which can be measured at 600nm. Similar to the DPPH assay, **TEAC** is based on the ability of molecules to scavenge the stable free radical of 2,2'- azinobis (3- ethylbenzothiozoline-6-sulfonic acid) and expressed as the Trolox equivalent (Badarinath et al., 2010).

Table 1.5 List of *in vitro* antioxidant evaluation method

S.No	Name of the method
I	Hydrogen Atom Transfer methods (HAT)
1)	Oxygen radical absorbance capacity (ORAC) method
2)	Lipid peroxidation inhibition capacity (LPIC) assay
3)	Total radical trapping antioxidant parameter (TRAP)
4)	Inhibited oxygen uptake (IOC)
5)	Crocin bleaching Nitric oxide radical inhibition activity
6)	Hydroxyl radical scavenging activity by p-NDA (p-butrisidunethyl aniline)
7)	Scavenging of H_2O_2 radicals
8)	ABTS radical scavenging method
9)	Scavenging of super oxide radical formation by alkaline (SASA)
II	Electron Transfer methods (ET)
1)	Trolox equivalent antioxidant capacity (TEAC) decolourization
2)	Ferric reducing antioxidant power (FRAP)
3)	DPPH free radical scavenging assay
4)	Copper (II) reduction capacity
5)	Total phenols by Folin-Ciocalteu
6)	N,N-dimethyl-p-Phenylenediamine (DMPD) assay

Source: Badarinath, Rao, Chetty, Ramkanth, Rajan, & Gnanaprakash, 2010.

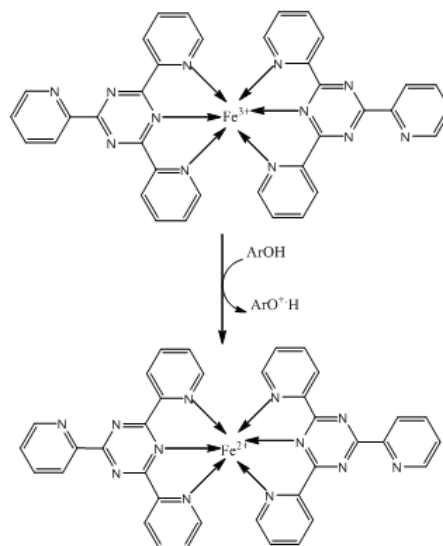
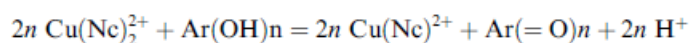


Figure 1.7 Reduction of Fe^{3+} -TPTZ to Fe^{2+} -TPTZ

Ferric reducing-antioxidant power (FRAP) assay It is based on the mechanism of reducing intense blue ferric tripyridyltriazine complex to the ferrous form as showed in

Figure 1.7 (Gupta, 2015). The reducing power of the target antioxidant could be reflected by the color change monitored under the wavelength of 593nm.

Cupric ion reducing antioxidant capacity (CUPRAC) CUPRAC method is designed to demonstrate the ability of scavenging hydroxyl radicals. The chromogenic oxidizing reagent, bis(neocuproine)copper(II) chloride [Cu(II)-Nc], reacts with polyphenols [Ar(OH)_n] (as shown in the equation below).



It is applicable to both hydrophilic and lipophilic and will not be affected by the sugars and citric acid commonly present in foods since it has a selective action on antioxidant compounds (Alam, Bristi, & Rafiquzzaman, 2013).

Folin-Ciocalteu method The reaction involves a chemical reduction of the mixture of tungsten and molybdenum oxides, in which, the molybdenum centre in the complex reagent is reduced from Mo (VI) to Mo (V), generating a blue color at 750 nm. The intensity of color is proportional to the concentrations of phenol compounds, thus, it is often used for the assessment of total phenolic content (Gupta, 2015).

Oxygen radical absorbing capacity (ORAC) assay Using Trolox as the reference, a test sample is applied to inhibit the oxidation of B-phycoerythrin initiated by thermal decomposition of azo compounds such as AAPH in the ORAC assay (Glazer, 1990). The peroxy radical reacts with a fluorescent probe to form a non-fluorescent product; thus, it reflects classical radical chain breaking antioxidant activity by H atom transfer (Ou et al., 2001).

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) Assay: In this method, the treatment of 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) with $K_2S_2O_8$ or MnO_2 provides a bluish-green radical cation (ABTS⁺) (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999). The ABTS radical cation could be further changed back to colorless neutral form in the presence of both lipophilic and hydrophilic compounds such as flavonoids, hydroxycinnamates and carotenoids (Gupta, 2015). Due to the high reactivity of ABTS radical cation, it could be scavenged by most antioxidants including phenolics, thiols and vitamin C (Walker & Everette, 2009).

Total radical trapping antioxidant parameter (TRAP) Assay Induced by AAPH (2'-azobis(2-amidinopropane) hydrochloride), the degree of peroxidation is monitored by screening of the loss of fluorescence from the protein R-phycoerythrin (R-PE) (Badarinath et al., 2010). It is especially good for the determination of *in-vivo* antioxidant capacity in serum or plasma since it measures nonenzymatic antioxidants (Huang, Ou, & Prior, 2005).

1.4.1.2 Model system

The existing spectrophotometric system is easy to carry out; however, direct analysis fails to predict the mechanism of antioxidants in inhibiting the oxidation in the complex food matrix (Frankel, 1993). Also, the free radicals used in each assay are non- physiological; therefore, it is difficult to capture the different modes of action of antioxidant. Currently, a few model systems such as ground meat, fish oil, cholesterol and β -carotene linoleate model systems have been developed (Rojas and Brewer 2007; Jayaprakasha et al. 2001; Shen et al., 2013; Zhang et al., 2013).

Meat model Since a big amount of iron could be released from denatured myoglobin and hemoglobin, they are able to catalyze the oxidation rapidly in cooked meats. Therefore, natural or synthetic antioxidants can be applied in the model for the evaluation of anti-lipid-oxidation capability through determining the production of malondialdehyde (MDA) (Rojas and Brewer, 2007).

Fish oil model The fish oil emulsion consists of water, lipid micelles, and other components which could mimic the biological fluids. Also, the ubiquitous polyunsaturated fatty acids (PUFAs) such as EPA and DHA would oxidize rapidly and are critical to indicate antioxidant efficacy. Thus, the results of these emulsion models could closely reveal the antioxidant power in preventing lipid oxidation in biological fluids and cells (Zhang et al., 2013).

Cholesterol model Cholesterol is the main components in the cell membrane and blood serum of mammals and can be oxidized under oxidative stress. The primary oxidized product of cholesterol is 7-ketocholesterol which is determined to predict the degree of emulsion oxidation. By comparing the efficiency of the antioxidant in retarding oxidation in the cholesterol emulsion, the result of the antioxidant potential would be more convinced (Shen et al., 2013).

1.4.2 *In vivo* methods

The *in vivo* studies usually use the test animals such as mice, rats, and rabbits etc. to evaluate the antioxidant effect of target food extract in specific organs or cells based on different administration and dose (Alam, Bristi, & Rafiquzzaman, 2013). After a certain period of time, the animals are sacrificed and blood or tissues are used for the determination.

Reduced glutathione (GSH) estimation GSH is an intra-cellular reductant and helps to protect cells against free radicals, peroxides and other toxic compounds in catalysis, metabolism and transport (Sapakal et al., 2008). As the irreplaceable role in transport system in the kidney, it is an essential indicator to reveal the *in vivo* antioxidant defense. The prepared sample supernatant is added with the Ellman's reagent (5,50-dithiobis-2-nitrobenzoic acid in phosphate buffer solution), then, the absorbance of the solution is read at 412 nm against blank after completion of the reaction (Alam, Bristi, & Rafiquzzaman, 2013).

Superoxide dismutase (SOD) method Similar to GSH, SOD is an enzyme which is able to remove the free-radicals caused by environmental adversity, and improve stress tolerance (Kong, Zhao, Liu, He, Tian, & Zhou, 2012). SOD dismutates the superoxide anion and thereby inhibits the reduction of a small hemeprotein cytochrome-c. Its antioxidant efficiency is estimated by an increase in absorbance recorded at 420 nm and expressed as units/mg protein (Alam, Bristi, & Rafiquzzaman, 2013).

Catalase (CAT) method

It is a tetrameric heme-containing enzyme that catalyzes the dismutation of H_2O_2 into water and oxygen molecules. It has high specificity for a high level of the presented H_2O_2 (Sharma, Jha, Dubey, & Pessarakli, 2012). The catalase activity of the hemolysate is determined by adopting the erythrocyte lysate method at 240 nm. One unit of activity represents the degradation of 1 mM of H_2O_2 /min and is expressed as units per milligram of protein (Alam, Bristi, & Rafiquzzaman, 2013).

Cell culture

A cellular antioxidant activity (CAA) assay was developed to evaluate the antioxidant

capability of bioactive compounds from food and plant products (Wolfe & Liu, 2007). Human hepatocarcinoma Hep-G2 cells are mixed with the redox sensor dihydrodichlorofluorescein (DCFH₂) to oxidized to fluorescent dichlorofluorescein (DCF) by ROO• radical induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH). It aims at measuring the ability to inhibit the intracellular DCFH₂ oxidation determined by fluorescence at λ_{exc} 485 nm and λ_{em} 535 nm. The obtained results are expressed in μ M of quercetin equivalents. Therefore, the CAA assay could be used for screening antioxidant activity in natural product extracts at the cellular level.

1.5 References

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CHAPTER 2. PHYTOCHEMICALS IN SWEET SORGHUM (*DURA*) AND THEIR ANTIOXIDANT CAPABILITIES AGAINST LIPID OXIDATION

2.1 Introduction

Sweet sorghum is widely cultivated around the world and utilized for food or animal feed (Afify et al., 2012). In the US, sorghum is mainly used for animal feed after the stalks are removed during post-harvest processing. Recently, sorghum millets have been found as an economical biofuel material for producing ethanol (Vasilakoglou et al., 2011). This potential application in bioenergy has raised the value of sorghum and led to its increasing production in the US annually. Although the color and texture of sorghum millets is not as desirable as other cereals such as wheat and oats for food applications, it has been reported to possess a variety of antioxidant phytochemicals including polyphenols, anthocyanins, carotenoids and tocopherols (Dykes & Rooney, 2006). Daily consumption of these antioxidants could help reduce the risk of developing chronic diseases associated with the oxidation of cholesterol and fatty acids (Parr, & Bolwell, 2003). For hydrophilic soluble antioxidants, they are usually extracted by water or alcohols and leave the major sorghum biomass intact for biofuel production. Therefore, the hydrophilic antioxidants extraction could be a pre-treatment in the biofuel production of sorghum. As the extract has great potential in health promoting application, the treatment can increase the economic value of sorghum used as a biofuel material.

The profiles and levels of phytochemicals in sorghum millets are dependent on the varieties and growth environments of sorghums. In this study, antioxidant

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phytochemicals and their antioxidant activity in the sweet sorghums harvested in Louisiana were identified and evaluated. The results would be helpful in utilizing Louisiana sorghum millets as a potential health promoting source and biofuel material at the meantime. To identify the major phytochemicals responsible for the activity and health benefits of sorghum millets, the hydrophilic and lipophilic phytochemicals in millets were extracted and evaluated individually. In addition to using common antioxidant activity assay methods, the antioxidant capabilities of both extracts were evaluated by using an emulsion model which consisted of cholesterol and linoleic acid at the same level as found in the human blood serum.

Cholesterol and fatty acids are the primary components in the cell membrane and blood serum of mammals and can be oxidized under oxidative stress. The oxidation of cholesterol and fatty acids results in generation of lipid oxidation products which are toxic to the endothelial cells of blood vessels and cell membranes. For example, 7-ketocholesterol, the primary cholesterol oxidation product could display pro-apoptotic, pro-inflammatory activities and cause degenerative diseases or lipid metabolism disorders (Biasi et al., 2009; Osada et al., 2012; Otaegui-Arrazola et al., 2010). The fatty acids, especially unsaturated fatty acids likely undergo lipid peroxidation and yield short chain inflammatory mediators, such as aldehydes and hydroperoxides which are associated with cellular damage, arthritis, cerebral arteries and other coronary diseases (Romero et al., 1998). Thus, inhibition of cholesterol or fatty acid oxidation in the serum is crucial to maintain normal lipid metabolism and integrity or permeability of cell membranes in the body. However, evidence of the function of antioxidant phytochemicals present in sweet sorghum millets in inhibiting cholesterol and fatty acid oxidation has not been

documented. The performance of antioxidants in sorghum millets in the cholesterol and linoleic acid emulsion model could closely reflect their antioxidant activity in the human blood serum.

2.2 Material and Methods

2.2.1 Chemicals and materials

HPLC grade methanol, hexane, acetic acid and sodium phosphate dibasic (anhydrous) were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Sodium phosphate monobasic (anhydrous) was obtained from Amresco (Solon, OH, USA). Isopropanol and sodium bicarbonate were obtained from Mallinckrodt Co. (Paris, KY, USA). Ethyl acetate and hydrochloride acid were purchased from EM Science (Gibbstown, NJ, USA). Tween 20, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox, Folin-Ciocalteu reagent, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), cholesterol, 7-ketocholesterol, linoleic acid, heptadecanoic acid (C17:0), boron trichloride in methanol (BCl₃-methanol), α -tocopherol, γ -tocopherol, apigeninidin-chloride and all the phenolic standards (ferulic acid, *p*-coumaric acid, cinnamic acid, catechin, gallic acid, syringic acid, kaempferol and quercetin) were provided by Sigma-Aldrich (St. Louis, MO, USA). Sweet sorghum millets (*Dura*) were obtained from the Sugar Research Station, Louisiana State University Agricultural Center (St. Gabriel, LA, USA).

2.2.2 Extraction and determination of the antioxidants in sweet sorghum millets

The extraction method was described in the study of Jang and Xu (2009). The sweet sorghum millets were ground using a kitchen blender. The ground powder was screened by using a 1 mm brass sieve. Ten grams of the sifted powder sample was extracted using 50 mL of acidified methanol (pH 1) twice at 60°C. The methanol layers were combined

and evaporated by a vacuum centrifuge evaporator (Labconco, Kansas City, MO, USA) to obtain the hydrophilic extract (HPE). The same procedure was applied to extract lipophilic compounds in the millet powder by using hexane solvent instead of methanol. Both hydrophilic and lipophilic extracts were weighed (dry weight basis) and used to prepare the stock solution (10 mg/mL) in methanol or hexane, respectively.

Anthocyanins and other phenolic compounds in the hydrophilic extract (HPE) were determined by the HPLC method described by Jang and Xu (2009). Carotenoids in the HPE were determined by the method as described by Sun et al (2007). Tocopherols in the lipophilic extract (LPE) were measured by using a normal phase HPLC system with a fluorescence detector. The HPLC analysis conditions were as described by Jang and Xu (2009).

2.2.3 Determination of total phenolic contents of the hydrophilic and lipophilic extracts

The total phenolic content method was described in the study of Jang and Xu (2009). Ten-fold diluted Folin-Ciocalteu reagent (0.75 mL) was reacted with 0.1 mL of the extract stock solution. After 5 min, 0.75 mL of sodium bicarbonate (60 g/L) was added to the mixture and incubated at 25°C for 90 min. The absorbance was recorded by a UV-visible SpectraMax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 750 nm. Catechin (0.05, 0.1, 0.2 mg/mL) was used to plot a standard curve. The total phenolic content of the extract was expressed as µg catechin equivalent /gram of sweet sorghum millets.

2.2.4 Determination of antioxidant activities of the hydrophilic and lipophilic extracts using DPPH method

The DPPH method was carried out using a modification of the procedure by Yue and Xu (2008). DPPH solution (0.1mM, 1.8 mL) was mixed with 0.2 mL of the hydrophilic extract stock solution or the lipophilic extract stock solution which was first evaporated to dryness and then re-dissolved by 0.2 mL of methanol. The reaction was carried out at 25°C for 30 min in the dark. The absorbance of the mixture at 515 nm was monitored at zero time (Ab_0) and after 30 min incubation (Ab_{30}), respectively. The inhibition rate was calculated as:

$$\text{Inhibition rate (\%)} = [(Ab_0 - Ab_{30}) / Ab_0] \times 100$$

Different concentrations of Trolox (0.05, 0.10, 0.20 and 0.50 mM) versus their corresponding inhibition rates were plotted to obtain a standard curve. The inhibition rate of the testing sample was calculated and converted to μmol Trolox equivalent /gram of sweet sorghum millets.

2.2.5 Preparation of cholesterol - linoleic acid oxidation emulsion

The cholesterol - linoleic acid oxidation emulsion consisted of cholesterol (1000 $\mu\text{g/mL}$), linoleic acid (500 $\mu\text{g/mL}$), AAPH (300 $\mu\text{g/mL}$), and Tween 20 (10 $\mu\text{L/mL}$) in phosphate buffer solution (PBS, pH 7.2). The emulsion was homogenized by a microfluidizer materials processor (M-110P, Microfluidics, Newton, MA, USA). The hydrophilic or lipophilic extract stock solution (40 or 80 μL) was added to a 40-mL vial and dried. Then, it was mixed and homogenized with 20 mL of the emulsion, resulting in the antioxidant-containing emulsion having either 20 or 40 $\mu\text{g/mL}$ of the extract. The emulsion without the extract served as a blank group. Each treatment and the blank group had three

replications. All the vials were incubated in a 37°C water bath and continually agitated by a multiple magnetic stirrer (Multistirrer, VELP Company, Italy) for 48h.

2.2.6 Extraction and determination of 7-ketocholesterol

One milliliter of the emulsion sample was collected from each vial at different time (0, 24 and 48 h) and mixed with 2 mL of hexane. The solution was vortexed and centrifuged at 5000 x g for 15 min. The upper hexane layer was separated and transferred to a clean test tube with 0.1 g of anhydrous NaSO₄. The primary oxidation cholesterol product, 7-ketocholesterol, was analyzed by an HPLC with normal phase column (id 250× 4.60 mm 5 micron, Supelco, Bellefonte, PA) and a UV detector. The mobile phase composition and gradient program were described in the studies by Xu et al (2005) and Zhang et al (2013). The inhibition rate of the extract for preventing cholesterol oxidation at each sampling time was calculated using the following equation:

$$\text{Inhibition rate (\%)} = [(C_b - C_t) / C_b] \times 100$$

where C_b was the concentration of 7-ketocholesterol in the blank at a sampling time; C_t was the concentration of 7-ketocholesterol in the treatment group at the same sampling time.

2.2.7 Extraction and determination of linoleic acid

One milliliter of the emulsion sample was collected and mixed with 2 mL of hexane containing internal standard C17:0 (100 µg/mL). The hexane supernatant was separated by centrifugation and transferred to a clean test tube. The hexane was evaporated by nitrogen flow to obtained dried sample. Two milliliters of BCl₃-methonal was added to the dried sample for esterification at 60°C for 30 min. Then, 1 mL of water and 1mL of hexane were mixed with the reaction solution and vortexed. After centrifugation, the

upper hexane layer was mixed with anhydrous NaSO₄ and transferred to a GC vial. The operating condition of GC analysis was as described by Zhang et al (2013). The retention rate of linoleic acid in the emulsion was calculated as follows:

$$\text{Retention rate (\%)} = (C_t / C_0) \times 100$$

where C₀ was the concentration of linoleic acid at 0 h; C_t was the concentration of linoleic acid at 24 or 48 h in the same emulsion.

2.2.8 Data analysis

The concentrations of bioactive components, total phenolic contents, and antioxidant activities of each extract were expressed as the mean and standard error from three independent extractions and analysis. The inhibition rate of cholesterol and retention rate of linoleic acid were expressed as the mean \pm standard error of three replications as well. All calculations were done using Microsoft Excel (Redmond, WA, US) and one-way ANOVA was used to evaluate significant differences between treatment means using the statistical analysis software (SAS, 9.1.3, Cary, NY, US).

2.3 Results and Discussion

2.3.1 Antioxidant phytochemicals in sweet sorghum millets

In addition to its protein, starch, and other macronutrients related to the body energy supply, sorghum millet offers a rich source of antioxidant phenolics with potential health benefits of preventing various chronic diseases. However, the profile and levels of phenolics in a crop usually varies by crop variety and growth environment (Afify et al., 2012). In this study, sweet sorghum (*Dura*) samples were harvested in Louisiana where annual average climate temperatures are much higher than in most traditional sorghum growing areas. The local environmental factors such as the higher average temperature

(25°C) or the average rainfall (5-6 inches/month) during growing season (May to October) may result in the antioxidant profiles and levels of Dura sorghum millets that differ from those harvested in cold and dry areas. In our study, the total phenolic content of HPE was 768.9 ± 46.7 µg of catechin equivalent/g of sorghum millets which was eight times higher than that of LPE (97.6 ± 8.2 µg of catechin equivalent/g of sorghum millets) (Table 2.1).

The dominant phenolic sorghum millets was apigeninidin at a level of 1.57 mg/g which is lower than that reported in black (4.0-9.8 mg/g) and red (3.3 mg/g) sorghum bran by Awika and Rooney (2004). The grain bran usually contains higher level of antioxidant phenolics than its kernels.

Table 2.1 Total phenolic content and DPPH free radical scavenging capability (TEAC) of hydrophilic (HPE) and lipophilic (LPE) extracts from the sweet sorghum millets

	HPE	LPE
Total phenolic content (µg of catechin equiv/g)	768.9 ± 46.7	97.6 ± 8.2
TEAC (µmol of trolox equiv/g)	6.5 ± 0.1	0.8 ± 0.1

Other phenolics such as ferulic, *p*-coumaric, cinnamic, gallic and syringic acid, catechin, kaempferol and quercetin were identified and quantified in the sorghum millet sample (Table 2.2). The level of catechin was 144.9 ± 3.7 µg/g and much higher than that in the millets reported (6.2-84.7 µg/g) by Bröhan et al (2011). Also, kaempferol, gallic and ferulic acid were the major phenolics in the sweet sorghum millets at levels of 133.7 ± 6.7 , 130.6 ± 8.1 and 107.6 ± 10.1 µg/g, respectively. In a previous study, the levels of gallic and ferulic acid ranged from 13.2-46.0 and 8.9-95.7 µg/g, respectively, in different sorghum varieties (Dykes & Rooney, 2006). The differences in phenolic composition and concentration of the sweet sorghum from other studies may be due to differences in

the light intensity, relative humidity, an average temperature of growing environment as well as the genetic background of the sorghum species. The combination of these phenolics has been reported to have pharmacological potential in preventing cell inflammatory and cardiovascular diseases (Calderon-Montano et al., 2011; Kalender et al., 2012). Although the carotenoids in fruits and vegetables and their health benefits in preventing retina-aging and prostate and breast cancer are widely reported, their levels and profile in sweet sorghum millets have not been well documented. Concentration of lutein and β -carotene in the sorghum millets was of 4.8 ± 0.2 and $18.8 \pm 1.1\mu\text{g/g}$, respectively (Table 2.2).

Table 2.2 The concentrations of bioactive components in sweet sorghum millets

Name	Concentration ($\mu\text{g/g}$)
Ferulic acid	107.6 ± 10.1
<i>p</i> -coumaric acid	17.9 ± 1.4
Cinnamic acid	8.9 ± 0.5
Catechin	144.9 ± 3.7
Gallic acid	130.6 ± 8.1
Syringic acid	38.8 ± 4.0
Kaempferol	133.7 ± 6.7
Quercetin	22.1 ± 0.9
Apigeninidin	1570.0 ± 9.3
Lutein	4.8 ± 0.2
β -carotene	18.8 ± 1.1
α -tocopherol	7.7 ± 0.7
γ -tocopherol	145.7 ± 12.7

2.3.2 Evaluation of antioxidant activities of the hydrophilic and lipophilic extracts

In the DPPH assay, the antioxidant activity of HPE was 6.5 ± 0.1 μmol of Trolox equivalent/g of sorghum millets, while it was only 0.8 ± 0.1 μmol of Trolox equivalent/gram of sorghum millets for LPE (Table 2.1). Although the DPPH assay is a common method for evaluating the antioxidant activity of phenolics in plant extracts, the

absorbance at 515 nm for measuring DPPH change is prone to the interference by other components, such as anthocyanins in the extract which has absorbance in that wavelength range as well. Thus, the DPPH method is not an ideal assay for determining the antioxidant activity of anthocyanins-rich extract. As the antioxidant activity determined by DPPH assay is the activity of quenching free radicals or H-donor capability of the antioxidant, it may not be actually associated with the required antioxidant function in the body. In fact, not only the activity of an antioxidant but also the properties of the surrounding media and lipid substrates could affect the performance of antioxidant capacity.

In our study, cholesterol and fatty acids, which were the key protecting targets of a health promoting antioxidant, were used as the substrates in the antioxidant activity evaluation model. Compared with DPPH assay, the activity obtained in this model is much correlated to the capability of the antioxidant in stabilizing lipids in a biological system and preventing hyperlipidemia stress. In this study, an *in vitro* cholesterol-linoleic acid emulsion model which simulated the blood serum environment was used to determine antioxidant activity of the sorghum millet extracts. Cholesterol is an important component of LDL and HDL in the serum. Cholesterol and linoleic acid are also the major lipids of the cell membrane. However, they are vulnerable to oxidation under oxidative stress or attack by free radicals. During oxidation, the generation and accumulation of cholesterol oxidation product 7-ketocholesterol, could lead to plaque formation in the blood vessel wall and development of coronary atherosclerosis (Tian et al., 2011). Also, linoleic acid could be oxidized to produce cytotoxic short chain aliphatic compounds (Spiteller, 1998). In the cholesterol-linoleic acid emulsion, an

increase in 7-ketocholesterol and decrease in linoleic acid were measured to assess the degree of cholesterol and fatty acid oxidation in the system, individually.

After 24 h oxidation, 7-ketocholesterol in the blank increased to 2.85 $\mu\text{g/mL}$ from undetectable levels (Figure 2.1). Meanwhile, the treatments with 20 and 40 $\mu\text{g/mL}$ of LPE had 7-ketocholesterol at 1.86 and 1.48 $\mu\text{g/mL}$, respectively. For the two HPE treatments, the level of 7-ketocholesterol was below 1 $\mu\text{g/mL}$. After 48 h oxidation, HPE showed higher antioxidant activity with an inhibition rate of 88.5% for HPE1 (20 $\mu\text{g/mL}$) and 92.2% for HPE2 (40 $\mu\text{g/mL}$), while it was 45.2 and 65.4% for LPE1 (20 $\mu\text{g/mL}$) and LPE2 (40 $\mu\text{g/mL}$), respectively (Figure 2.1). For linoleic acid oxidation, only 33.6% of linoleic acid was retained in the blank after 24 h oxidation. The retention rate from low to high was in the order of LPE1 40.1%, LPE2 45.7%, HPE1 61.7%, and HPE2 83.3% (Figure 2.2). After 48 h oxidation, the retention rate of linoleic acid in the blank was only 15.5%. HPE2 still exhibited the highest retention rate (70.4%) compared to HPE1 (52.8%), LPE1 (22.0%), and LPE2 (33.6%). Although both HPE and LPE exhibited inhibiting activity against cholesterol and linoleic acid oxidation in a dose-dependent property, HPE had higher antioxidant activity than LPE in the emulsion model. The abundant apigeninidin and other phenolics could be responsible for the high antioxidant activity of HPE. Most of the hydrophilic phenolics possess at least two hydroxyl groups that could greatly contribute to the protection of lipid substrates from oxidation. The results (Figure 2.1) are agreement with the study of Zhang et al (2013). In that study, hydrophilic anthocyanins extract had a higher capability in inhibiting cholesterol oxidation than tocopherols extract. Since apigeninidin has multiple cyclic rings and its molecular structure is similar to that of cholesterol, it may have better accessibility than

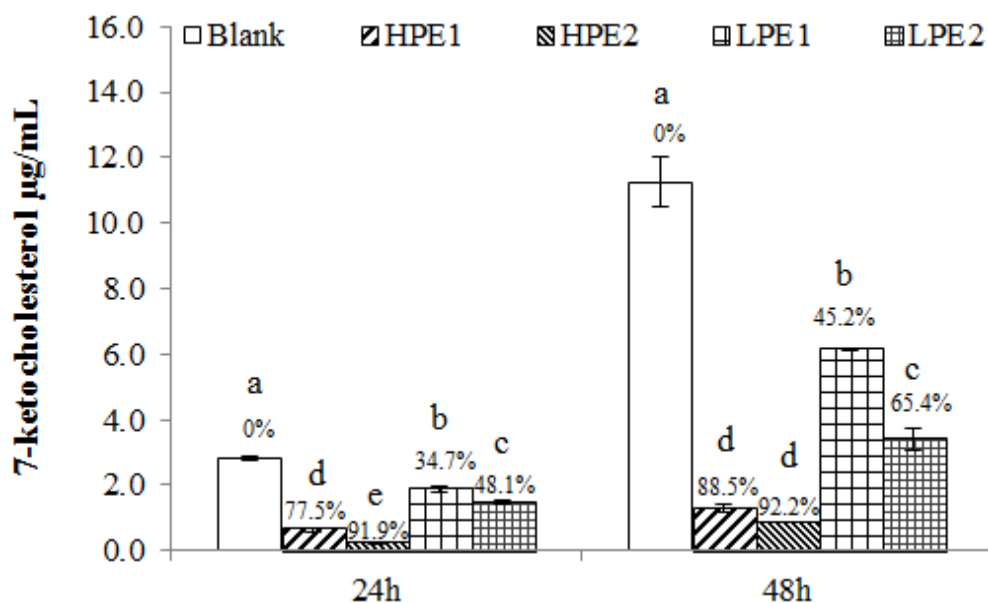


Figure 2.1 The 7-ketocholesterol levels and cholesterol oxidation inhibition rates in blank, HPE1 (20µg/mL), HPE2 (40µg/mL), LPE1 (20µg/mL) and LPE2 (40µg/mL) after 24 and 48h oxidation.

*Different letters represent significant differences among the five treatments at $P < 0.05$.

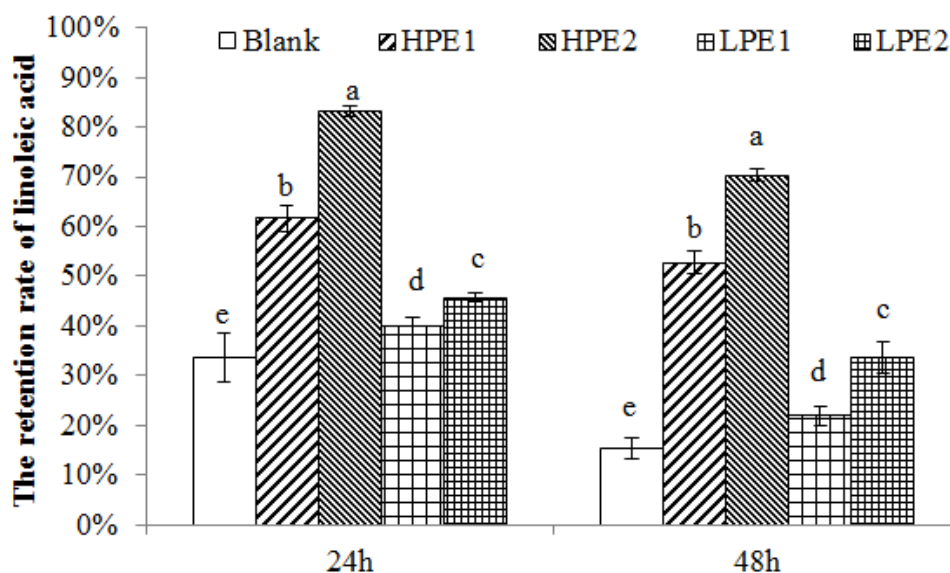


Figure 2.2 The retention rates of linoleic acid in blank, HPE1 (20µg/mL), HPE2 (40µg/mL), LPE1 (20µg/mL) and LPE2 (40µg/mL) after 24 and 48h oxidation.

*Different letters represent significant difference among the five treatments at $P < 0.05$.

other long carbon chain antioxidants in contacting and protecting cholesterol against oxidation. Although LPE had a high level of γ -tocopherols, the lower level and diversity of other phenolics in LPE were responsible for the lower overall capability in stabilizing lipid. In general, HPE played an important role in preventing lipid oxidation in the emulsion system. In other words, the antioxidant capacity of the sweet sorghum millets is mainly contributed by the hydrophilic phenolic antioxidants.

2.4 Conclusion

In this study, the levels and profiles of hydrophilic and lipophilic antioxidant phytochemicals in sweet sorghum millets were evaluated. The dominant hydrophilic and lipophilic antioxidant was apigeninidin and γ -tocopherol, respectively. The hydrophilic antioxidants showed higher antioxidant activity than lipophilic antioxidants in both the DPPH assay and cholesterol-linoleic acid oxidation model. Therefore, the health benefits of sweet sorghum may be mainly attributed by its hydrophilic antioxidant phytochemicals. Thus, with its high biomass and diverse antioxidants, sweet sorghum (*Dura*) could be a valuable biomaterial for both biofuel and health promoting applications.

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CHAPTER 3. COMPARISON OF PHENOLIC PROFILES AND ANTIOXIDANT POTENTIALS OF THE LEAVES AND SEEDS OF THAI HOLY AND SWEET BASILS

3.1 Introduction

Basil, originating from India and other regions of Asia, is well known for its distinct aroma which was mainly contributed by 1,8-cineole, methyl cinnamate, methyl chavicol, and linalool (Lee *et al.*, 2005; Klimánková *et al.*, 2008). Among nearly 150 varieties, Thai holy basil (*Ocimum sanctum* Linn.) and Thai sweet basil (*Ocimum basilicum* var.*thyrsiflora*) leaves were commonly used as flavorful additives in Thai cuisine such as stir-fried dishes and spicy soups (Juntachote *et al.*, 2007). On the other hand, the basil seed extract could be used in preparing edible films, or emulsifying solutions because of abundant polysaccharides (Khazaei *et al.*, 2014).

Recently, the aromatic compositions of basils have been widely studied, while it is also noted for its antioxidant values. The determination of phytochemical phenolics present in basils is important in studying the relationship with some health promoting functions, including protection of cells from damage inflicted by free radicals, improvement of immune system, and anti-inflammatory properties (Zhang *et al.*, 2013). For example, it has been reported that basil leaves were promising immunostimulants which could increase the serum protein and globulin in the blood of the fish; this was considered as a sign of improvement in both specific immune response and non specific immune responses (Nahak & Sahu, 2014). However, the current information of

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antioxidant capability of basil leaves or seeds was relatively general and limited in the results of total phenolic content, reducing power, DPPH free radical scavenging activity, superoxide anion and hydrogen peroxide assays (Yeşiloğlu & Şit, 2012). There was no study on comparing phenolic profiles and the lipid or cholesterol oxidation inhibition capabilities of both seeds and leaves of basil. The information is very important to understand the antioxidation and health promoting function mechanisms of basil.

It is well known that reactive oxygen species (ROS) and free radicals could be produced by the potential environmental pollutions or unhealthy living habits such as smoking or consumption of lipid-oxidized food (Lushchak, 2011). Thus, the latent adverse compounds in environment and oxidized food products could act as inflammatory mediators, invade the body circulation system and accelerate atherothrombotic and other cardiovascular diseases (Sun *et al.*, 2005). Generally, various synthetic and natural antioxidants have been widely used to inhibit lipid oxidation in food products and assist to prevent the development of pathological events associated with lipid oxidation in body (Sgherri *et al.*, 2010). However, the long-term consumption of synthetic antioxidants in food products may cause potential health hazards (Lobo *et al.*, 2010). Thus, except the flavor enhancing characteristic, basil leaves may be an edible plant with existing ideal antioxidant properties to extend food shelf life and reduce the risks of lipid-oxidation-related diseases in human body (Shimizu *et al.*, 2013; Abo *et al.*, 2008).

To our knowledge, this is the first study reporting the anti-cholesterol-oxidation efficiency of the selected basil in an emulsion model. Also, the phenolic profile and antioxidant potential of basil seeds and leaves were evaluated and compared. In general,

the results obtained from this study revealed the abundant phenolics and effective anti-lipid-oxidation capability of common Thai basil. The information could be useful for utilizing the basil or its extract as a food preservative or potential health promoting ingredient in food products.

3.2 Materials and Methods

3.2.1 Chemicals and materials

HPLC grade acetonitrile, methanol, hexane, acetic acid and sodium phosphate dibasic (anhydrous) were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Sodium phosphate monobasic (anhydrous), isopropanol and sodium bicarbonate were obtained from Amresco (Solon, OH, USA) and Mallinckrodt Co. (Paris, KY, USA), respectively. Tween 20, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azobis(2-methylpropionamidine) dihydrochloride (AAPH), Trolox, Folin-Ciocalteu reagent, cholesterol, 7-ketocholesterol and the phenolic standards (rosmarinic, caftaric, chicoric, protocatechuic, caffeic, *p*-coumaric and *p*-hydroxybenzoic acid, rutin, and catechin) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thai holy and sweet basil leaves and seeds were harvested from a local horticultural garden during summer season (July) (Baton Rouge, LA, USA).

3.2.2 Extraction and determination of antioxidants in basil leaves and seeds extracts

Fifty grams of Thai holy basil (*Ocimum sanctum* Linn.) or Thai sweet basil (*Ocimum basilicum* var. *thyrsiflora*) leaves and seeds were freeze dried and then ground. Five grams of the ground sample was then mixed with 10 mL of methanol and incubated in a

60°C water bath for 30 min prior to extraction. After centrifuged for 20 min, the supernatant of the extraction mixture was collected. The extraction was repeated twice. Both of the supernatants were combined and evaporated to dryness. Each dried extract was prepared as a stock solution of 100 mg/mL in methanol.

3.2.3 Identification and determination of individual phenolic by using HPLC

One milliliter of the diluted basil leaves or seeds extract stock solution (20 mg/mL) was transferred to an HPLC vial. The determination was carried out by using an high performance liquid chromatography system (2690, Waters, Torrance, USA) with a C18 column (id 250×4.60 mm 5micron, Phenomenex, Torrance, USA) and a photodiode array detector. The HPLC running condition was based on the study of Du *et al* (2014). The phenolics were identified and quantified by comparing the retention times and spectrums and calibration curves obtained from their corresponding standards, respectively.

3.2.4 Total phenolic content

Folin- Ciocalteau method was used for the measurement of TP content based on the description in the study of Du *et al* (2014). The Folin reagent (0.75 mL) was diluted ten times with distilled water and mixed with 0.1 mL of the diluted stock solution (5 mg/mL). Then, 0.75 mL of sodium bicarbonate (60 g/L) was added to the mixture and incubated at 25 °C for 90 min. The absorbance was measured at 750 nm by an UV-visible SpectraMax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA). The TP content of each extract was expressed as µg catechin equivalent (CE)/g dry weight (DW) based on the calibration curve of catechin.

3.2.5 Scavenging DPPH free radical activity

The scavenging DPPH free radical ability was determined by using the procedure with minor modification in the study of Yue & Xu (2008). DPPH solution (0.1 mM, 1.8 mL) was mixed with 0.2 mL of each diluted basil extract stock solution (5 mg/mL) and incubated in dark for 30 min at 25 °C. The absorbance before (Ab_0) and after (Ab_{30}) the incubation were recorded at 515 nm, respectively. The scavenging DPPH free radical ability was expressed as μmol Trolox equivalent (TE)/g dry weight (DW) based on the calibration curve of Trolox.

3.2.6 Evaluation of anti-lipid-oxidation capability by using cholesterol emulsion model

The cholesterol emulsion which contained 1% cholesterol, 0.1% of Tween 20 and 2% of AAPH was homogenized by using a microfluidizer materials processor (M-110P, Microfluidics, Newton, MA, USA). The Thai holy basil or sweet basil leave and seed extract (250 $\mu\text{g/mL}$ of the emulsion) was mixed with 20 mL and prepared as the treatment group, respectively, while the emulsion without any extract was used as a control group. Each of the emulsions was placed in a 40 mL vial and incubated in a 37°C water bath and continually agitated by a multiple magnetic stirrer (Multistirrer, VELP Company, Italy) for 144h. The major cholesterol oxidation product 7-ketocholesterol in the emulsion was determined every 48h to assess the degree of cholesterol oxidation by the HPLC method described in the study of Zhang *et al* (2013).

3.2.7 Data analysis

The levels of phenolics, total phenolic contents, scavenging free radical activities and the anti-lipid-oxidation capability were expressed as the mean \pm standard error from three independent determinations. All the data was calculated by using Microsoft Excel

(Redmond, WA), and one-way analysis of variance ANOVA (SAS, 9.1.3, Cary, NY) was used to evaluate significant differences at $p < 0.05$.

3.3 Results and Discussion

3.3.1 Phenolic profiles of the basil leaves and seeds

In this study, a total of eight primary phenolics including rosmarinic, caftaric, chicoric, protocatechuic, caffeic, *p*-coumaric and *p*-hydroxybenzoic acid and rutin were identified and determined in the Thai holy and sweet basil leaves and seeds (Figure 3.1). Chicoric acid was the most dominant phenolic in both THBL and TSBL (Table 3.1). Compared with ten commercial basil varieties with chicoric acid levels ($\mu\text{g/g DW}$) ranging from 64.8 ± 0.3 to 162.4 ± 0.1 in the study of Lee & Scagel (2010), THBL and TSBL contained much higher concentrations of 439.85 ± 5.28 and 224.17 ± 0.34 , respectively (Table 3.1). Although rosmarinic acid has been consistently noted as the primary phenolic acid in most basil varieties, it was about 1.5 and 2 times lower than chicoric acid in THBL and TSBL, respectively (Table 3.1) (Lee & Scagel, 2010; Hakkim *et al.*, 2007). Also, the following caftaric acid ($\mu\text{g/g DW}$) at the level of 202.18 ± 0.66 for THBL and 59.00 ± 2.31 for TSBL was more abundant than the ten selected basil samples ranging from 22.8 to 3.5 $\mu\text{g/g DW}$ in the study of Lee & Scagel (2010). The abundant phenolic profile of the basil in this study might be due to the optimal growing condition where average temperature in summer was 25 to 32°C with sufficient sunlight. Shiga *et al.* (2009) stated that it may be possible to increase the phenolic content of sweet basil by manipulating light conditions.

However, caffeic acid was only identified in Thai holy basil. It has been reported that caftaric acid was the secondary metabolites of caffeic acid in plants (Crozier

et al., 2006). The higher amount of caftaric acid and lower amount of caffeic acid in basil leaves could be explained that most caftaric acid was derived from caffeic acid

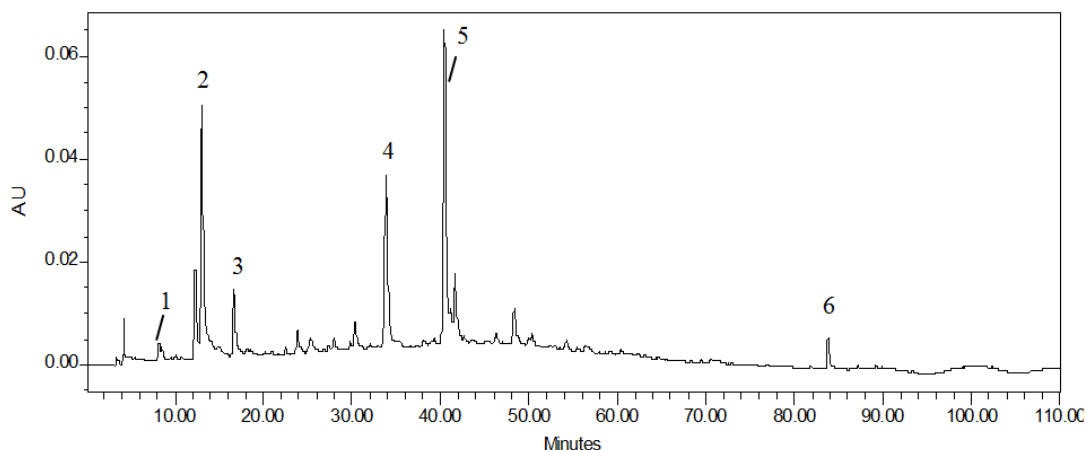


Figure 3.1 A typical chromatogram of phenolics in Thai holy basil leaves: 1, protocatechuic acid; 2, caftaric acid; 3, caffeic acid; 4, chicoric acid; 5, rosmarinic acid; 6, *p*-hydroxybenzoic acid.

during growth by phytochemical and microbial conversion (Wu *et al.*, 2007). Thus, relatively high level of caftaric acid was found in THBL, while its caffeic acid was much lower than that of THBS (Table 3.1). Based on various *in vivo* or *in vitro* studies, the phenolics, chicoric, rosmarinic, caftaric and caffeic acids have been confirmed to provide various health promoting functions (Khair-ul-Bariyah *et al.*, 2012). For example, chicoric acid was reported to prevent hyperglycemic and obesity through attenuating hepatic steatosis, while, rosmarinic, caffeic and caftaric acid were responsible for anti-inflammatory, antitumor and antimutagenicity functions, respectively (Zhang *et al.*, 2011; Xiao *et al.*, 2013; Boonyarikpunchai *et al.*, 2014). Additionally, compared with other studies on the phenolic profile of different basil varieties, protocatechuic and *p*-hydroxybenzoic acids were only detected in Thai sweet and holy basil leaves (Kwee &

Niemeyer, 2011). The protocatechuic acid, which has been evidenced to offer antiatherosclerotic, hyperlipidemic and cardiac protective functions, was approximately twice higher in TSBL than in THBL (Table 3.1) (Borate, Suralkar, Birje, Malusare, & Bangale, 2011). Thus, as a culinary seasoning, the basil leaves could not only enhance food flavors but also potentially provide health benefits.

Table 3.1 Phenolic profiles of Thai holy and sweet basil leaves and seeds

Phenolics ($\mu\text{g/g DW}$)	Thai Holy Basil		Thai Sweet Basil	
	Seeds	Leaves	Seeds	Leaves
Rosmarinic Acid	$17.00 \pm 0.27\text{c}$	$303.83 \pm 4.55\text{a}$	N.D.	$116.18 \pm 4.11\text{b}$
Caftaric Acid	N.D.	$202.18 \pm 0.66\text{a}$	N.D.	$59.00 \pm 2.31\text{b}$
Caffeic Acid	$200.52 \pm 8.80\text{a}$	$70.22 \pm 2.79\text{b}$	N.D.	N.D.
Chicoric Acid	$27.57 \pm 1.25\text{c}$	$439.85 \pm 5.28\text{a}$	N.D.	$224.17 \pm 0.34\text{b}$
<i>p</i> -Hydroxybenzoic Acid	$32.52 \pm 2.20\text{b}$	$52.09 \pm 0.64\text{a}$	$22.59 \pm 5.27\text{c}$	$26.06 \pm 0.60\text{c}$
<i>p</i> -Coumaric Acid	$59.32 \pm 2.18\text{a}$	N.D.	N.D.	N.D.
Protocatechuic Acid	$47.70 \pm 2.49\text{c}$	$81.14 \pm 0.66\text{b}$	N.D.	$152.64 \pm 1.80\text{a}$
Rutin	$24.66 \pm 2.25\text{a}$	N.D.	$26.26 \pm 5.08\text{a}$	N.D.
Overall Level	409.29	1149.31	48.85	578.05

*Mean values with different letters are significantly different ($p < 0.05$).

*N.D. not detected.

For basil seeds, the information of their phenolic profiles is very limited. Generally, basil seeds were processed into essential oil products or prepared as the thickening and stabilizing ingredient in food system because of the abundant polysaccharides (Rafe *et al.*, 2012). In this study, THBS had caffeic acid which was the dominant phenolic and at a level above 200 $\mu\text{g/g DW}$. The following phenolics were

chioric, *p*-coumaric, protocatechuic, and *p*-hydroxybenzoic acid with the concentrations ranging from 27.57 ± 1.25 to 59.32 ± 2.18 $\mu\text{g/g DW}$ (Table 3.1), while the level of rosmarinic acid and rutin was below 25 $\mu\text{g/g DW}$. However, TSBS only contained *p*-hydroxybenzoic acid and rutin with the concentration of 22.59 ± 5.27 and 26.26 ± 5.08 $\mu\text{g/g DW}$, respectively. The overall levels of phenolics in THBL, TSBL, THBS and TSBS were 1149.31, 578.05, 409.29 and 48.85 $\mu\text{g/g DW}$, respectively. THBL had the greatest diversity and highest overall level of phenolics.

3.3.2 Total phenolic contents and scavenging DPPH free radical activities of the basil leaves and seeds extracts

Basil variety had a statistically significant effect on the TP content of leaves which was based on the reducing power contributed by the compounds in the sample. For example, the TP content ($\mu\text{g CE/g DW}$) was significantly higher in TSBL (304.01 ± 12.46) than THBL (212.05 ± 4.90) while it was below 10 ($\mu\text{g CE/g DW}$) in THBS and TSBS. In a previous study, TP content of the selected basil was only 20.25 ± 0.85 $\mu\text{g CE/g DW}$ which was fifteen and ten times lower than that of TSBL and THBL, respectively (Kim *et al.*, 2011). Even compared with some related herb species which TP content ranged from 7.78 ± 0.31 to 108.28 ± 7.11 $\mu\text{g CE/g DW}$, the TP contents of TSBL and THBL was still much higher (Kim *et al.*, 2011). However, the TP contents of the basil extracts were not consistent with their overall phenolic levels. For instance, TSBL had significantly higher TP content than THBL. However, the overall phenolic level in TSBL was twice lower than that of THBL (Table 3.1 & Figure 3.2). The disparity of TP content and overall phenolic level in the basil extracts may be due to some interference such as reducing

sugar in TP content assays (Table 3.1 & Figure 3.2). Except phenolics, other non-antioxidant compounds such as reducing sugar, or aromatic amines in a test sample could also exhibit reducing power and contribute to the total phenolic content (Dai & Mumper, 2010). As Thai sweet basil has significantly higher sugar content than Thai holy basil, the higher TP content in TSBL may be due to its high reducing sugars responsible for the sweet taste of the basil (Luthria *et al.*, 2006).

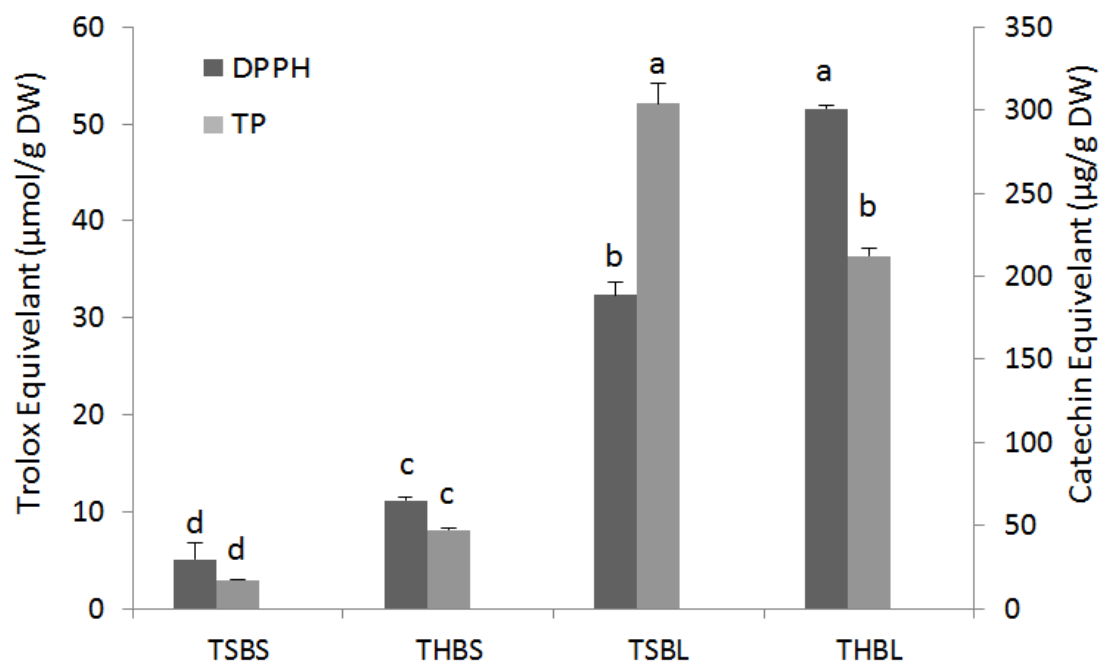


Figure 3.2 Scavenging DPPH free radical activities and total phenolic contents of Thai holy and sweet basil leaves and seeds (n=3). DPPH or TP content bars with different letters indicate significant difference ($P < 0.05$).

The DPPH assay reflects the scavenging metastable free radicals capability (DPPH) which could incorporate with the hydrogen radicals from potential antioxidants (Kwee & Niemeyer, 2011). In this study, the free radical quenching activity of each basil extract was correspondingly in the same order with the overall phenolic level rather than TP content (Table 3.1 & Figure 3.2). In general, the basil leaves had greater performance

than the seeds in scavenging DPPH free radicals since most phytochemical synthesis occurs in the leaves during plant growth (Rosa *et al.*, 2009). THBL dominated in the DPPH assay with the free radical scavenging activity of $51.56 \pm 0.35 \mu\text{mol TE/g DW}$ (Figure 3.1). It was at the similar level as Genovese basil ($54.0 \mu\text{mol TE/g DW}$), but significantly higher than Dark Opal or Sweet Thai basil in the study of Nguyen *et al* (2010). The following TSBL was 37% lower than THBL in scavenging DPPH free radicals activity, however, it was approximately three and five times higher than THBS and TSBS, respectively (Figure 3.2). The free radical quenching ability of THBL and THBS were approximately twice and one and a half times higher than the white and red holy basil in the study of Wangcharoen & Morask (2007), respectively.

3.3.3 Capability of basil leaves and seeds extracts in inhibiting cholesterol oxidation

Apart from the DPPH and TP content assays, a cholesterol emulsion model was established to provide complementary insight into the antioxidant capacity of basil leaves and seeds. The level of 7-ketocholesterol in the emulsion was determined by a normal phase HPLC method to predict the degree of cholesterol oxidation and antioxidant efficiency of each extract. At the beginning of 48h, the four treatments and control group generated a similar level of 7-ketocholesterol (approximately $0.5 \mu\text{g/mL}$) (Figure 3.3). Among all the four treatments, TSBL and THBL exhibited the greatest anti-lipid-oxidation capabilities. Their concentrations of 7-ketocholesterol were below $1.0 \mu\text{g/mL}$ after 96h without significant difference (Figure 3.3). However, it was $7.18 \pm 0.71 \mu\text{g/mL}$ after 96h in the control group which was twice higher than that in THBS and TSBS

(Figure 3.3). After 144h oxidation, TSBL and THBL contained much lower levels of 7-ketocholesterol at 4.5 ± 0.3 and 2.7 ± 0.3 $\mu\text{g/mL}$, respectively.

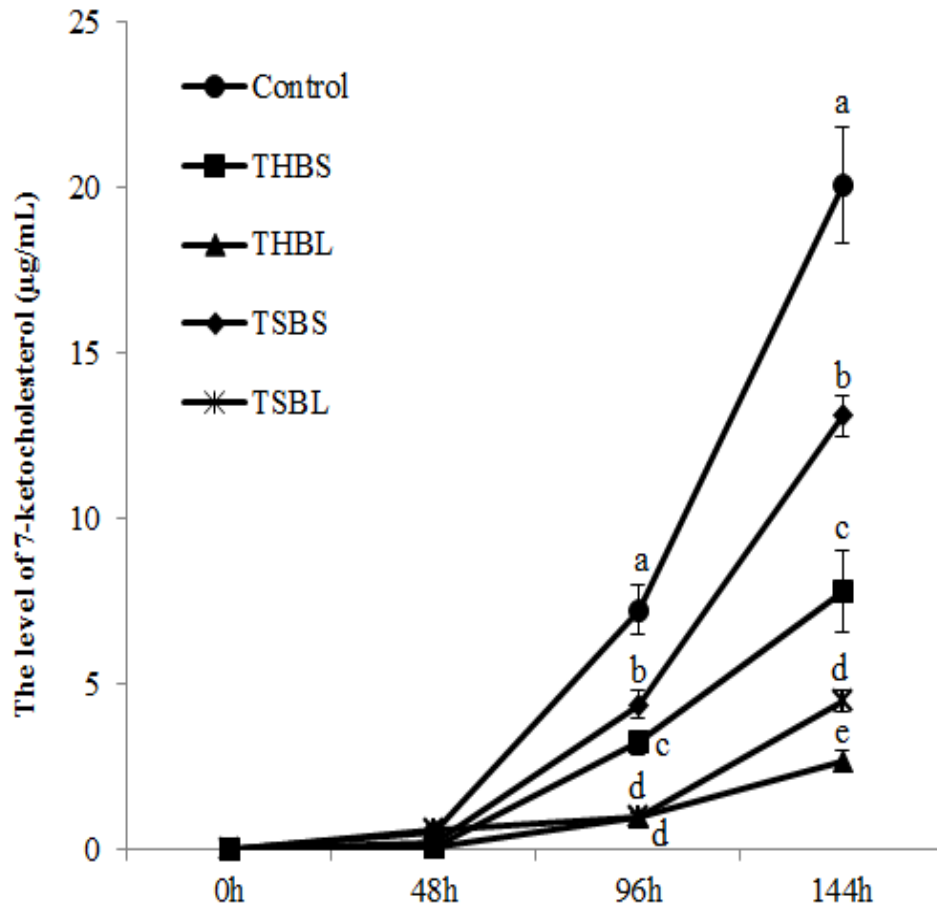


Figure 3.3 Capabilities of inhibiting 7-ketocholesterol cholesterol oxidation product of Thai holy and sweet basil leaves and seeds (n=3). Values at the same sampling time with different letters are statistically different ($P < 0.05$).

It is well known that the free radicals are ubiquitous and could initiate and accelerate oxidative stress which further causes food deterioration or excessive free radical injury in human bodies (Lobo *et al.*, 2010). In order to simulate the oxidative environment, AAPH was used in this study to generate free radicals through interacting with carbon-centered radicals and molecular oxygen (Yokozawa *et al.*, 2000). Then, the yielded peroxy radicals from the reaction could attack other lipid molecules to form lipid

hydroperoxide and new radicals repeatedly and result in the damage in physical or chemical alterations, cellular damage and a diverse array of pathological changes (Yokozawa *et al.*, 2000). In this study, the presence of basil leaves extracts in the cholesterol emulsion demonstrated effective inhibition capability against the cholesterol oxidation and production of 7-ketocholesterol. It was in accordance with the results obtained in DPPH assay (Figure 3.2) and the overall phenolic contents (Table 3.1). The capability of inhibiting cholesterol oxidation was correspondingly in the same order with the overall phenolic level of each basil extract rather than its TP content. The abundant phenolics in basil leaves were responsible for inactivating the catalytic cations or scavenging free-radical chain reactions.

Compared with the seeds, the basil leaves contained at least five phenolics including rosmarinic, caftaric, chicoric, *p*-hydroxybenzoic and protocatechuic with O-dihydroxy structure in the B ring which is regarded as the key site to contribute to the great antioxidative potential (Yokozawa *et al.*, 2000). Similarly, although the phenolic diversity in Thai holy basil seeds was similar to its leaves, the relatively low concentrations and the lack of important chicoric and caftaric acids which are associated with anti-inflammatory effects made the seeds not as competitive as the leaves (Lee & Scagel, 2009; Casanova *et al.*, 2014). Thus, the combination of multiple phenolics and higher levels of certain phenolics with great lipid oxidation suppression capability could provide synergistic and efficient antioxidant effect.

Additionally, based on the concentration of 7-ketocholesterol, either Thai holy basil leaves or seeds had better performance in inhibiting cholesterol oxidation than Thai sweet basil. For instance, THBS had twice lower level of 7-ketocholesterol than TSBS at

144h which may result from the poor diversity and low overall level of the phenolics in TSBS. On the other hand, despite both types of basil leaves having 7-ketocholesterol below 5.0 µg/mL, the oxidation product in THBL was twice lower than that in TSBL. Except for protochatchuic acid, other phenolic acids determined in the Thai holy basil leaves were several folds higher than those in Thai sweet basil leaves, especially the top two important phenolics, chicoric and rosmarinic acid (Table 3.1). For example, rosmarinic acid contains a caffeoyl group which has been reported to contribute to the potent superoxide anion scavenging activity by an electron spin resonance (ESR) method (Yagi *et al.*, 2002). Also, rosmarinic acid was evidenced to spontaneously penetrate membranes to inhibit lipid peroxidation and prevent the alteration of lipid membranes by oxidative stress and reduce the atherogenic index in an *in vitro* study (Fadel *et al.*, 2011). In fact, the number, orientation, and distance of active caffeoyl group have important influence on the molecular antioxidant activity. Therefore, the diaxial conformations of the two caffeoyl residues in chicoric acid could maximize their interactions in scavenging free radicals and prevent lipid oxidation according to the study of LeBlanc *et al* (2012).

Additionally, based on a study of high-fat-diet-mice, chicoric acid was found to lower serum lipid parameters and attenuate hepatic inflammation as well as exhibited hypoglycemic activity on type-2 diabetic rats (Xiao *et al.*, 2013). Thus, the enhanced antioxidant performance of Thai holy basil leaves than those of Thai sweet basil leaves might be due to the abundant levels and profile of the phenolics. The result obtained from the cholesterol emulsion model could not only be helpful to understand the anti-lipid-oxidation capabilities of Thai holy and sweet basil, but also provide useful

information linking their antioxidant capability with maintaining the body healthy antioxidative status and preventing various lipid-oxidation related diseases.

3.4 Conclusions

In general, the phenolic profiles of Thai holy and sweet basil seeds and leaves were determined in this study. This is the first study to investigate the anti-lipid-oxidation capabilities of Thai holy and sweet basil seeds and leaves by using a cholesterol emulsion model. In this study, rosmarinic, caftaric, chlorogenic, *p*-hydroxybenzoic and protocatechuic acid were detected in both THBL and TSBL. Caffeic acid was only found in Thai holy basil. Additionally, THBS had seven primary phenolics including rosmarinic, caffeic, chlorogenic, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic acid and rutin, while only *p*-hydroxybenzoic and rutin were found in TSBS. In DPPH assay, THBL dominated in scavenging free radical activity and was followed by TSBL, THBS and TSBS. The order of TP content from high to low TSBL, THBL, THBS and TSBS was not consistent with their order in anti-lipid-oxidation and scavenging free radicals activities. These activities were consistent with their overall levels and diversities of phenolics in the basil extracts. Thai holy basil leaves exhibited greater anti-lipid-oxidation performance than its seeds or Thai sweet basil leaves and seeds. Therefore, Thai holy basil leaves could be processed as an antioxidant ingredient to be applied in various food products. In our further study, animal and cell system will be applied to verify the health promoting function of the Thai holy basil leaves.

3.5 References

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CHAPTER 4 BIOACTIVES IN HYDROPHILIC AND LIPOPHILIC BUTTERFLY PEA (*CLITORIA TERNATEA*) SEEDS AND PETALS EXTRACTS AND THEIR CAPABILITIES IN INHIBITING HEP-2 CARCINOMA CELLS PROLIFERATION

4.1 Introduction

Butterfly pea (*Clitoria ternatea*), a member of Fabaceae family and Papilionaceae sub-family, is a perennial leguminous twiner (Al-Asmari et al., 2014). Approximately 60 butterfly pea species are distributed within the tropical belt while a few species can be found in temperate areas (Al-Asmari et al., 2014). The abundant dietary anthocyanins in butterfly pea petal make it as popular health prompting tea and natural blue colorant in a variety of foods (Mukherjee et al., 2008). Also, triterpenoids, flavonol glycosides, alkaloids were reported in butterfly pea leaves, while, pentacyclic triterpenoids, taraxerol and taraxerone were identified in the roots (Singh & Tiwari, 2010). Since butterfly pea is both rainfall and drought tolerant and self-pollinated, it is easy to harvest a large quantity of its seeds (Morris, 2009). However, information of the composition of the seeds, especially its health promoting constituents such as phenolics, tocopherols, and phytosterols was limited. Thus, the hydrophilic and lipophilic phytochemicals in the butterfly pea seeds and petals were identified and quantified in this study.

Except for the antioxidant and antibacterial function in food application, the pharmacognostic performance of anti-diabetes, nootropic, anxiolytic, anti-convulsant, sedative, antipyretic, anti-inflammatory and analgesic in different parts of butterfly pea

also have been investigated (Jain & Shukla, 2011). However, the anti-cancer potential of butterfly pea petal and seed has not been well documented. Since laryngeal carcinoma accounts for 25% of head and neck carcinoma, it becomes the second most common respiratory cancer following lung cancer (Mirunalini et al., 2011). The limitless replication of cancer cells and multiple interactions with their microenvironments increase the difficulties in cancer treatments (Pienta et al., 2008). Primary clinical treatments for laryngeal cancer include surgery, chemotherapy and radiotherapy, however, they would induce adverse side effects or even result in resistance to these therapies (Agostinis et al., 2011). Therefore, attention has recently been devoted to plant-derived compounds which have pharmacological functions on tumor with minimum side symptoms (Veerabadran et al., 2013). In order to evaluate the anticancer capabilities of butterfly pea petal and seed, the effect of hydrophilic and lipophilic extracts on inhibition of laryngeal cancer cell (HEp-2) proliferation was studied. Therefore, the results of this study not only provide specific phytochemical profile in both butterfly pea petal and seed, but also are very helpful to understand the connections between the bioactive compounds and anti-cancer potential.

4.2 Materials and Methods

4.2.1 Chemicals and materials

HPLC grade acetonitrile, acetic acid, methanol, and hexane were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Acetone was purchased from Macron (Charlotte, NC,

USA). Ethyl acetate was purchased from EM Science (Gibbstown, NJ, USA). trimethylsilyl imidazole (TMSIM), BCl₃-methanol and phenolics, fatty acids and tocopherol standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fresh butterfly pea seeds and petals (*Clitoria ternatea*) were obtained from a local garden in Baton Rouge, LA, USA. The human carcinoma HEp-2 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Other reagents and culture media including fetal bovine serum (FBS), antibiotic (penicillin–streptomycin), Cell Titer Blue, dimethyl sulfoxide (DMSO), and phosphate buffered saline (PBS) were purchased from Invitrogen (Grand Island, NY, USA).

4.2.2 Extraction of hydrophilic and lipophilic bioactive compounds in butterfly pea seeds and petals

The freeze dried butterfly pea seeds and petals were ground by a coffee blender (Hamilton Beach, Southern Pines, NC, USA). The hydrophilic compounds of petals or seeds (20 g) was obtained by the extraction of using 50 mL of methanol at 60°C for 20 min. After centrifugation, the methanol layer was transferred to a clean tube. Then, the solid residues was extracted two more times at the same condition and procedure. The dried hydrophilic extract was obtained by evaporating the methanol using a vacuum centrifuge evaporator (Labconco, Kansas City, MO, USA). The dried extracts were combined and prepared for a stock solution (50 mg/mL in methanol). As for the lipophilic extract of butterfly pea petals or seeds, 50 mL of a mixture of ethyl acetate and

hexane (50:50; v:v) was used to carry out the extraction at the same procedure used for obtaining the hydrophilic extract. After the solvent extract was dried, a stock solution of the lipophilic extract (50 mg/mL in hexane) was prepared as well.

4.2.3 Identification and quantification of hydrophilic and lipophilic phytochemicals and fatty acids

Phenolics Phenolic profiles of the hydrophilic extracts were determined by a reversed phase HPLC (2690, Waters, Torrance, USA) coupled with C18 column (id 250×4.60 mm, 5 micron, Phenomenex, Torrance, USA), a diode array detector and the operation condition as same as in the study of Du et al. (2014). The concentrations of major phenolics were calculated based on their corresponding standard curves. Both of the extracts were also subject to an LC-MS analysis to identify the compounds without their reference standards available. The LC-MS consisted of a UPLC apparatus (Thermo Scientific Dionex UltiMate 3000, Waltham, MA, USA) and a MS (Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™) which had an electrospray ionization source (ESI) and was operated in the positive mode with a full MS scan from 150 to 2000 m/z. The LC-MS separation was carried out using a reversed phase column (Acclaim® Mixed-Mode WAX-1, 150 × 2.1 mm, 5 µm). The mobile phase consisted of A (1% formic acid solution) and B (acetonitrile) at a constant flow rate of 0.2 mL/min with a gradient program of 0 - 70% B at 0.0 - 8.0 min, 70 - 100% B at 8.0 - 10.0 min, 100 - 0% B at 10 - 15 min. The MS parameters were set as: electric potential of the ESI source, 3.0 kV;

capillary temperature, 300 °C; heater temperature, 200 °C. The concentrations of ternatins and delphinidin derivative were calculated by the standard curve of cyanidin chloride in molar concentration and then converted to mg/g of sample based on its molecular weight.

Tocopherols A normal phase HPLC (1100 series, Agilent, Santa Clara, CA, USA) with Supelcosil LC-Si column (id 250× 4.60 mm 5micron, Supelco, Bellefonte, PA, USA) was used for the determination of tocopherols. The condition of the HPLC analysis was the same as the method described in Jang and Xu (2009).

Fatty Acids each of the lipophilic extracts was reacted with 2 mL of BCl₃-methanol to carry out methylation after 200 µg of internal standard (C17:0) was added. The methylation reaction mixture was incubated at 60°C for 30 min. Then, 1 mL of water and 1 mL of hexane were added to the reaction solution and vortexed. After centrifugation, the upper hexane layer was transferred to a clean test tube and mixed with anhydrous NaSO₄ to remove any moisture before it was transferred to a GC vial. The fatty acids were determined by a GC equipped with FID detector with a Supelco SP2380 (30 m x 0.25 mm) column (Bellefonte, PA, USA). The GC condition was as same as that in the study of Yue et al (2008).

Phytosterols The determination of phytosterols was based on the study of Xu and Godber (1999) with minor revision. After the lipophilic extract was mixed with 20 µg cholesterol internal standard, it was dried and incubated with 200 µL of TMS and 50 uL

of acetonitrile at 65 °C for 30 min. The derived products were extracted with 200 uL of hexane followed by a GC-MS analysis. A Varian CP-3800 GC (Valnut Creek, CA, USA) coupled with a Saturn 2200 mass spectrometer and a DB-5 column (L60 m × i.d. 0.25 mm and 0.25 µm thin coating film) (Supelco, Bellefonte, PA, USA) was used in the analysis. The initial oven temperature was 200°C, then, ramped to 280 °C at an rate of 10°C/min and held at the final temperature for 62 min. Helium was the carrier gas at a constant flow rate of 1.5 mL/min. The injection port temperature and a split ratio were set at 280°C and 1:50, respectively. The peak area ratios of different levels (5, 10, 20, 50 and 100 µg) of campesterol, stigmasterol, and β-sitosterol to 20 µg of cholesterol internal standard were used to set up a standard curve for quantifying the phytosterols.

4.2.4 Determination of capability of inhibiting human carcinoma cell (HEp-2) proliferation

The human carcinoma HEp-2 cell line was used to assess the anticancer capability of hydrophilic extracts of butterfly pea seed (HBS) and petal (HBP) and lipophilic extracts of butterfly pea seed (LBS) and petal (LBP). The cell line was cultured in Dulbecco's modified eagle's media (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin–streptomycin) and grown in a 5% CO₂ atmosphere with 95% humidity at 37 °C for 24h. Then, the cells were harvested, counted (3×10^4 cells/ml), and transferred into a 96-well plate. The working solution of each extract was prepared by dissolving HBS, HBP, LBS, or LBP with 0.2 % DMSO in PBS culture

media. The HEp-2 cells were incubated with a series of concentrations of HBS or HBP working solution (1.0, 0.5, 0.25, 0.12 and 0.06 mg/mL) and LBS or LBP working solution (12.0, 9.0, 6.0, 3.0 and 1.5 mg/mL) for 96 h at 37°C for a dose dependent study. The cells only mixed with the media and 0.2% DMSO were used as the control. For measure the cell viability, the media was discarded and replaced with 100 µL fresh media containing 20% Cell Titer Blue. After the cells were stained for 4 h, fluorescence intensity of the media was read at excitation/emission wavelengths of 570/615 nm using a FluoStar Optima micro-plate reader (BMG, Germany). The anticancer capability of the extract at each concentration treatment was expressed by a survival rate which was the percentage of the viable cells in the treatment versus the control.

4.2.5 Data analysis

The determination of hydrophilic or lipophilic phytochemicals and fatty acids in each extract was repeated in triplicate and expressed as means \pm standard deviation. The significant differences between the components in two lipophilic extracts were determined by one-way ANOVA at $P < 0.05$ (SAS, 9.1.3, Cary, NY, USA). The determination of anticancer capability of each treatment concentration or control was repeated five times and analyzed by GraphPad Prism (version 6.0, GraphPad Software Inc., USA). The differences between the treatments and control were analyzed by two-way ANOVA at $P < 0.05$.

4.3 Results and Discussions

4.3.1 Hydrophilic and lipophilic phytochemicals and fatty acids in butterfly pea seeds and petals

The yields of the hydrophilic extracts, HBS and HBP, were 3.66 and 4.05 %, respectively.

The chromatograms of the hydrophilic phytochemicals in HBS and HBP are shown in Figure 4.1 and 4.2, respectively. In addition abundant ascorbic acid (1.32 ± 0.02 mg/g FW), sinapic acid was the dominant phenolic among the fifteen major hydrophilic phenolics at a concentration of 1.01 ± 0.07 mg/g FW followed by epicatechin and gallic acid in the seeds (Table 4.1). Compared with the contents in rapeseeds (0.09 - 0.59 mg/g FW) and camelina seeds (0.39 mg/g FW) (Nićiforović & Abramović, 2014), the sinapic acid content in butterfly pea seeds was much higher than each of them (Table 4.1). It was reported that sinapic acid could assist to suppress the expression of proinflammatory mediators via NF- κ B inactivation in regulating inflammatory status and immune response (Yun et al., 2008). Epicatechin, a proanthocyanidin, in the butterfly pea seeds which was reported to exhibit immunoregulatory, anti-hypertension effects as well was ten times higher (0.56 mg/g FW, Table 4.1) than that reported in the garden pea seeds (*Pisum sativum*) (0.05 mg/g) (Ferraro et al., 2014; Litterio et al., 2015). Protocatechuic, *p*-coumaric, rutin and two hydroxycinnamic acid derivatives in the butterfly pea seeds were all above 0.30 mg/g FW, while kaempferol, apigenin, caffeic, syringic, ferulic, rosmarinic and cinamic acid were in a range of 0.04 to 0.22 mg/g FW (Table 4.1).

Compared with the rutin content in edible *Amaranthus* seeds which was between 0.0711 and 0.0775 mg/g DW) (Li et al., 2015), the content in butterfly pea seeds was much higher and reached 0.31 mg/g FW (Table 4.1).

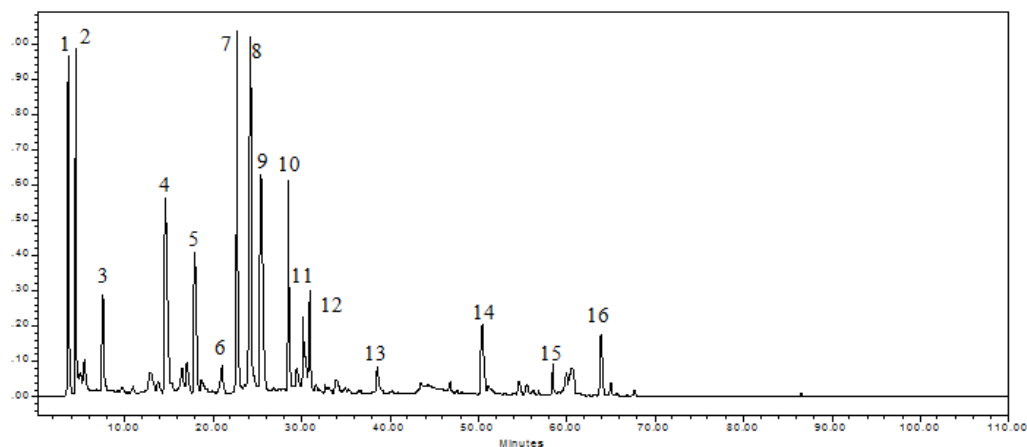


Figure 4.1 Chromatogram of the hydrophilic extract of butterfly pea seeds

1 – Vitamin C; 2 – Gallic acid; 3 – Protocatechuic acid; 4 – Epicatechin; 5 – Caffeic acid; 6 – Syringic acid; 7 – Sinapic acid; 8 – Hydroxycinnamic acid derivatives; 9 – *p*-coumaric acid; 10 – Hydroxycinnamic acid derivatives; 11 – Rutin; 12 – Ferulic acid; 13 – Rosmarinic acid; 14 – Cinnamic acid; 15 – Kaemferol; 16 – Apigenin.

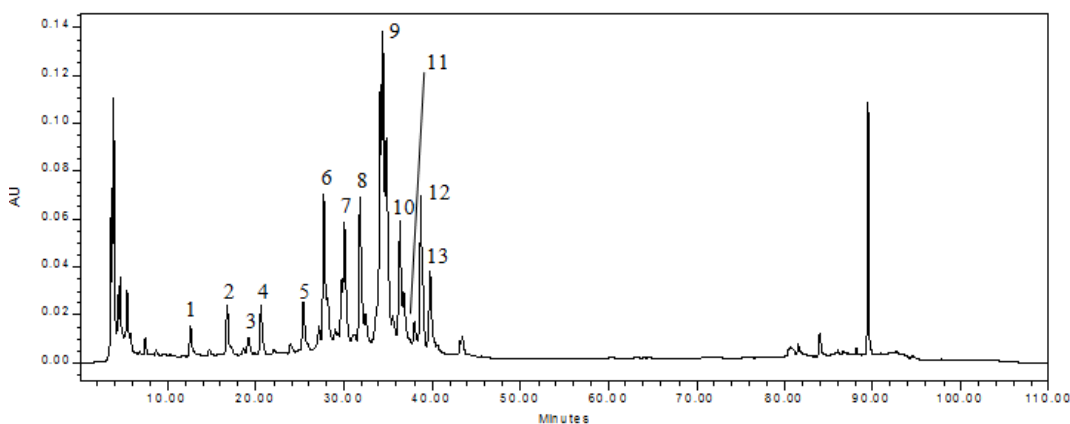


Figure 4.2 Chromatogram of the hydrophilic extract of butterfly pea petals

1 – Cyanidin-3-sophorose; 2 – Delphinidin derivative; 3 – Ternatin A1; 4 – Ternatin B3; 5 – Ternatin D3; 6 – Ellagic acid; 7 – Rutin; 8 - Delphinidin derivative; 9 – Kaempferol-3- neohesperidoside; 10 – Quercetin-3-(2G-rhamnosylrutinoside); 11 – Ternatin B2; 12 – Ternatin C2; 13 – Ternatin D2.

Although mung bean, radish, broccoli and sunflower seeds contain gallic, protocatechuic, caffeic, *p*-coumaric, ferulic, chlorogenic, sinapic acid, quercetin and kaempferol (Pająk et al., 2014), each of their levels was significantly lower than that in the butterfly pea seeds.

Table 4.1 Bioactive compounds in the hydrophilic extract of butterfly pea seeds

Peak No.	Compounds	Concentration (mg/g FW)
1	Ascorbic acid	1.32 ± 0.02
2	Gallic acid	0.42 ± 0.00
3	Protocatechuic acid	0.34 ± 0.01
4	Epicatechin	0.56 ± 0.03
5	Caffeic acid	0.22 ± 0.01
6	Syringic acid	0.14 ± 0.01
7	Sinapic acid	1.01 ± 0.07
8	Hydroxycinnamic acid derivative 1	0.57 ± 0.19
9	<i>p</i> -Coumaric acid	0.30 ± 0.01
10	Hydroxycinnamic acid derivative 2	0.44 ± 0.02
11	Rutin	0.31 ± 0.01
12	Ferulic acid	0.15 ± 0.00
13	Rosmarinic acid	0.05 ± 0.00
14	Cinamic acid	0.08 ± 0.00
15	Kaempferol	0.04 ± 0.00
16	Apigenin	0.09 ± 0.00

The unique phytochemicals in butterfly pea petals are ternatins, a group of polyacylated delphinidin derivatives (Sasaki et al., 2013). It was reported that Ternatin A1-A3, B1-B4, C1-C5 and D1-D3 consist of delphinidin 3, 3', 5'-triglucoside attached with malonic acid, glucose, *p*-coumaric acid or caffeic acid (Kazuma et al., 2003). In this study, ternatin C2 and D2 in the butterfly pea petals were observed at higher concentrations of 1.81 ± 0.09 and 1.45 ± 0.07 mg/g FW, respectively. The concentrations of ternatin A1, B3, D3 and

B2 were in a range of 0.32 to 0.51 mg/g FW (Table 4.2). The chemical structures of the six types of ternatins are elucidated in Figure 4.3. Two delphinidin derivatives and

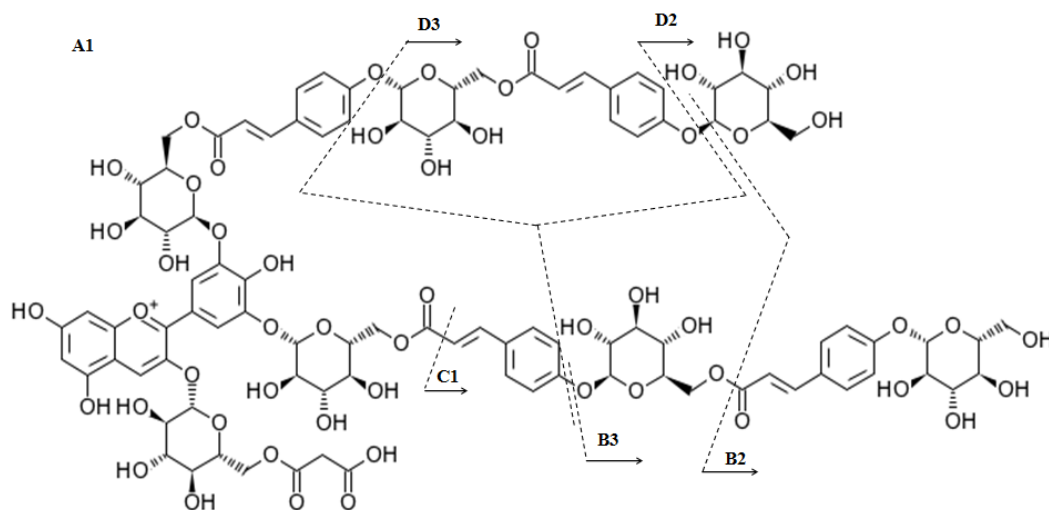


Figure 4.3 Chemical structures of Ternatin A1, B2, B3, C2, D2 and D3

Table 4.2 Hydrophilic bioactive compounds in the hydrophilic extract of butterfly pea petals

Peak No.	Compounds	Concentration (mg/g FW)
1	Cyanidin-3-sophoroside	0.31 ± 0.02
2	Delphinidin derivative	0.28 ± 0.01
3	Ternatin A1	0.51 ± 0.03
4	Ternatin B3	0.50 ± 0.03
5	Ternatin D3	0.54 ± 0.01
6	Ellagic acid	0.21 ± 0.01
7	Rutin	0.89 ± 0.04
8	Delphinidin derivative	2.13 ± 0.16
9	Kaempferol 3-neohesperidoside	1.76 ± 0.05
10	Quercetin 3-(2G-rhamnosylrutinoside)	0.37 ± 0.01
11	Ternatin B2	0.32 ± 0.01
12	Ternatin C2	1.81 ± 0.09
13	Ternatin D2	1.45 ± 0.07

cyanidin-3-sophoroside were also identified and responsible for the blue color of the petals together with ternatins. In general, kaempferol 3-neohesperidoside, quercetin 3-(2G-rhamnosylrutinoside) and rutin were the major flavanol glycoside compounds in the petals, while ellagic acid was the only phenolic acid identified in the butterfly pea petals (Table 4.2).

The yields of the lipophilic extracts, LBS and LBP, were 5.28 and 0.80 %, respectively. Lipophilic phytosterols are chemically characterized as triterpenes and considered to be the structural components of plant cell membranes (Moreau, Whitaker, & Hicks, 2002). Similar to the function of cholesterol in animal cells, free phytosterols serve to stabilize phospholipid bilayers in the plant cells (Moreau, Whitaker, & Hicks, 2002). Although several studies investigated phytosterols in *Clitoria Ternatea* species, most of them only focus on leaves, roots or petals (Kapoor & Purohit, 2013). In this study, β -sitosterol (40.17 ± 3.73 mg/100g FW) in the butterfly pea seeds extract LBS was significantly higher than that of the petals extract LBP (6.77 ± 0.19 mg/100g FW) (Table 4.3). It was reported that the level in butterfly pea roots and shoots was between 6 and 9 mg/100g (Kapoor & Purohit, 2013). Also, the seeds extract LBS contained campesterol at a level of 8.07 ± 0.22 mg/100g FW which was several times higher than that of the petals extract LBP (1.24 ± 0.02 mg/100g FW) (Table 4.3) and some fruit or vegetable seed such as pepper seeds (4.23 - 5.41 mg/100g FW) and tomato seeds (1.08 - 6.56 mg/100g FW) (Silva et al., 2013; Gupta et al., 2015). However, the levels of stigmasterol

in butterfly pea seeds and petals were similar and 7.95 ± 0.63 and 6.70 ± 0.83 mg/100g FW, respectively (Table 4.3) and approximately five and eight times higher than in berryfruit (0.50 - 1.60 mg/100g FW) and pepper seeds (0.63 - 0.93 mg/100g FW) (Silva et al., 2013; Salvador et al., 2015). Phytosterols were confirmed to possess hypocholesterolic function and reduce the risks of benign prostatic hyperplasia, cardiovascular diseases, colon and breast cancer development, as well as immunological effects in macrophages (Hamed et al., 2014).

Table 4.3 Lipophilic bioactive compounds in the lipophilic extracts of butterfly pea seeds and petals

	Compounds	Seeds	Petals
Fatty Acid (mg/g FW)	Palmitic acid (C16:0)	$3.61 \pm 0.13b$	$2.13 \pm 0.18a$
	Stearic acid (C18:0)	$2.85 \pm 0.15b$	$1.99 \pm 0.16a$
	Petroselinic acid (C18:1n6c)	$1.55 \pm 0.10b$	$1.01 \pm 0.04a$
	Linoleic acid (C18:2n6c)	$8.73 \pm 0.61b$	$4.72 \pm 0.51a$
	Arachidic acid (C20:0)	$0.46 \pm 0.03b$	$0.36 \pm 0.01a$
	Behenic acid (C22:0)	$0.41 \pm 0.02b$	$0.30 \pm 0.03a$
	Phytanic acid	N.D.	$0.81 \pm 0.06a$
Phytosterol (mg/100g FW)	Campesterol	$8.07 \pm 0.22b$	$1.24 \pm 0.02a$
	Stigmasterol	$7.95 \pm 0.63a$	$6.70 \pm 0.83a$
	β -Sitosterol	$40.17 \pm 3.73b$	$6.77 \pm 0.19a$
	Sitostanol	$5.10 \pm 0.05b$	$1.20 \pm 0.03a$
Tocols (mg/100g FW)	α -Tocopherol	$0.17 \pm 0.06a$	$0.20 \pm 0.01a$
	γ -Tocopherol	$5.44 \pm 0.30b$	$0.24 \pm 0.02a$

*N.D. not detected

**Concentrations in each row with different letters are statistically different at $p < 0.05$.

As for the tocol contents, the butterfly pea seeds had abundant γ -tocopherol (5.44 ± 0.30 mg/100g FW) compared with the grape seeds (14.1 - 30.2 mg/kg) and Jatropha

curcas seeds (33.9 mg/kg) reported in the studies of Sabir et al (2012) and Corzo-Valladares et al (2012), respectively. However, γ -tocopherol in the butterfly pea petals was twenty times lower than that in the seeds (Table 4.3). The levels of α -tocopherol in butterfly pea seeds and petals were similar and 0.17 ± 0.06 and 0.20 ± 0.06 mg/100g FW, respectively (Table 4.3). These tocopherols have been evidenced for protecting cell membrane against reactive lipid radicals and prevention of atherosclerosis and carcinogenesis (Yang et al., 2013).

For the fatty acids profile, both of the butterfly pea seeds and petals had palmitic acid (C16:0), stearic acid (C18:0), petroselinic acid (C18:1), linoleic acid (C18:2) and arachidic acid (C20:0) and behenic acid (C22:0) (Table 4.3). Among them, linoleic acid was the most abundant fatty acid and had 8.73 ± 0.61 and 4.72 ± 0.51 mg/g FW in the butterfly pea seeds and petals, respectively. It is well known that linoleic acid is an essential fatty acid and required for assisting normal biological activities in the brain and heart (Blanchard et al., 2013). Besides, palmitic, stearic and petroselinic acids were all above 1.0 mg/g in the seeds and petals (Table 4.3). Different from the results of a previous study (Mukherjee et al., 2008), arachidic and behenic acids were first time observed in the butterfly pea in this study (Table 4.3). Furthermore, phytanic acid was found in the butterfly pea petals and might be derived from a microbial breakdown of chlorophyll to release phytol followed by further oxidation to phytanic acid (Jansen &

Wanders, 2006). In the study of Jansen & Wanders (2006), phytanic acid was involved in several mechanisms for regulating, triglycerides/cholesterol status in skeletal muscles.

4.3.2 Capabilities of hydrophilic and lipophilic butterfly pea seeds and petals extracts in inhibiting carcinoma cell (HEp-2) proliferation

Different concentrations of the four extracts HBS, HBP, LBS and LBP in media were prepared and used to treat HEp-2 cells to carry out a dose dependent study for each of them. HBS was the most effective extract against the survival rate of HEp-2 cells which rapidly decreased from 100.0 to 7.2% at the level of HBS ranging from 0 to 0.25 mg/mL (Figure 4.4). However, the survival rates of HEp-2 in HBP treatment still remained 90.6 - 100.0 % at the same concentration range (Figure 4.4). As the concentration of HBP increased from 0.25 to 0.50 mg/mL, the survival rate of HEp-2 then rapidly reduced to 17.2 % (Figure 4.4). Both HBP and HBS could inhibit 95% of the HEp-2 cell proliferation after the concentration was increased to 1.0 mg/mL (Figure 4.4). On the other hand, the inhibition effect was observed in LBS and LBP treatment when their concentrations had to be increased to 1.5 mg/mL. There was no significant difference between the two treatments with the concentration lower than 6 mg/mL (Figure 4.4). At a concentration of 9 mg/mL, LBS exhibited approximately 2.5 times higher capability than LBP in inhibiting HEp-2 cell growth. Compared with the lipophilic extracts of seeds and petals, the hydrophilic extracts had much higher capability in inhibiting carcinoma proliferation (Figure 4.4).

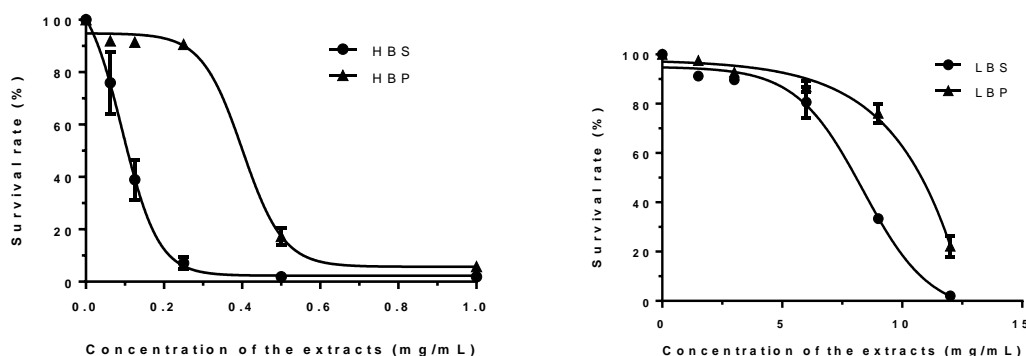


Figure 4.4 The survival rates of Hep-2 cells treated by different concentrations of the hydrophilic (HBS and HBP) and lipophilic (LBS and LBP) extracts of butterfly pea seeds and petals

Generally, Krebs Cycle is the primary metabolic pathway for providing ATP for normal cell growth with the help of mitochondria (Wen, Zhu, & Huang, 2013). Different from normal cells, cancer cells inevitably rely on metabolic reprogramming and undergo glycolytic pathway to realize rapid energy generation and macromolecular synthesis because of mitochondria dysfunction (Suh et al., 2013). The high glycolytic fluxes in cancer cells further induces apoptosis in neighborhood normal cells, blocks immune system and induces tissue invasion by tumors (Suh et al., 2013). Thus, the inhibition of glycolysis is a biochemical basis for designing therapeutic strategies to preferentially inhibit cancer cells with minimal residual systemic toxicity. Since the butterfly pea seeds contained abundant phenolics, they might act individually or synergistically to block the key enzyme in glycolytic metabolism in cancer cells and inhibit cancer cells proliferation. For example, the anti-cancer proliferation mechanism was based on the non-covalent interactions between cellular proteins and phenolic compounds (Aslan, Guler & Adem,

2015). Phenolic compounds such as rutin, quercetin , kaempferol, catechin, *p*-coumaric, sinapic, ferulic, syringic, caffeic and gallic acid which were determined in butterfly pea seed have been proved to affect important control point enzyme (pyruvate kinase isoenzyme M2) or attack glucose transporters (GLUT) in glycolytic pathway and regulate cancer cell production (Aslan, Guler & Adem, 2015). Thus, it may explain the reason that HBS exhibited the highest efficiency among the four types of extracts in inhibiting HEp-2 cell proliferation. Furthermore, various anthocyanins such as ternatins and cyanidin glycoside could be the primary bioactive compounds in HBP for contributing to its anticancer capability. This finding was in agreement with the results obtained in the studies of Wang and Stoner (2008) that anthocyanin extracted from black raspberries could counteract cancer cell motility through disruption of an essential mediator cyclooxygenase-2 (COX-2) in tumorigenesis. However, Dai et al. (2009) suggested that anthocyanin extract alone could less contribute to anticancer ability but may act additively or synergistically with other active components in inhibition of cancer cell growth which explain the possible reason that HBS had greater anti-cancer potential than HBP.

Tocopherols and phytosterols were the primary bioactive compounds in LBP and LBS. As reported by Yu et al. (2009), the anticancer actions of γ -tocopherol involved in death receptor 5 (DR5) protein upregulation, which could further stimulate tumor

necrosis and restrict its proliferation. Among the four determined phytosterols, β -sitosterol has been evidenced as the most effective one in inhibiting the growth of cancer cells via activation of certain enzymes which in turn induce cellular apoptosis (Bradford & Awad, 2007). Woyengo et al. (2009) suggested that β -sitosterol and campesterol could alleviate cancer development by reducing the production of carcinogens in biological metabolism. The levels of β -sitosterol and campesterol in LBS was approximately seven and eight times higher than those in LBP, respectively. Thus, it may be the reason why LBS had better performance than LBP in inhibiting HEp-2 cell growth in the concentrations ranging from 5 to 12 mg/mL.

As discussed above, certain phenolic compounds, tocopherols and phytosterols could demonstrate inhibitory efficiency by targeting specific enzymes or interfering metabolic pathway of cancer cells without affecting other non-tumorigenic counterparts and cells. Similar results were discovered that normal human epidermal keratinocytes and astrocytes were able to survive when exposed to flavonoids or green tea leaves rich in polyphenols, while they elicited the death of tumor cells (Hsu et al., 2003; Das et al., 2010). Therefore, the butterfly pea seed or petal extracts, especially the seeds hydrophilic extract, could possibly be an efficient supplement which has anticancer function without the adverse side effects as chemotherapeutic drugs and therapy.

4.4 Conclusion

The concentration and profile of phenolics, tocopherols, phytosterols and fatty acids in butterfly pea seeds and petals were determined. HEp-2 carcinoma cell line was used to evaluate the anticancer capability of butterfly pea seeds and petals extracts (HBP, HBS, LBP and LBS). The results indicated HBS was the most efficient in inhibiting the viability of HEp-2 cells. Both of HBS and HBP exhibited greater performance than LBS and LBP in inhibiting the cells proliferation. Therefore, the hydrophilic and lipophilic butterfly pea seeds and petals extracts possess health promoting function and could be used as a functional food ingredient or potential cancer therapy supplement.

4.5 References

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CHAPTER 5 THE CHANGE OF MICRONUTRIENTS IN SWEET POTATO AND THE EFFECT OF DIFFERENT EXTRACT FRACTION ON PC-12 CELL PROLIFERATION AFTER FERMENTATION WITH *LACTOBACILLUS ACIDOPHILUS* LA-K

5.1 Introduction

Sweet potato is the root of *Ipomoea batatas* (L.) Lam. (Convolvulaceae) originated from the Central America, an important industrial crop, and a good source of useful dietary fiber and vitamins (Kim et al., 2013). It has been considered to be one of the most promising economic crops due to its versatility, high production yield and strong resistance for various environmental conditions (Wu et al., 2015). As sweet potato can provide necessary nutrients in daily food supplies, it has been concerned by more and more people. In order to obtain the maximum absorption of the bioactive compounds, sweet potato is usually boiled, steamed, baked or roasted before consumption. However, the negative correlation between thermal processing and the impairment of the functional compounds of sweet potato has been widely reported (Tang, Cai & Xu, 2015). Currently, fermentation becomes a popular processing method which provides the preservation and organoleptic properties for the food such as cheese, vinegar, wine and fermented soybean with desirable sensory, aromas and textures (Kwon, Nyakudya & Jeong, 2014). Sweet potato has been fermented for the production of bioethanol; however, characterization and bioactivities of different fractions of sweet potato extracts have not been well documented (Masiero, Peretti, Trierweiler, & Trierweiler, 2014).

Generally, most bioactive compounds are in a bound form in the sweet potato flesh and they would be gradually metabolized in intestine which contains probiotic floras helping the releasing and biotransformation of those health benefit compounds via fermentation (Kubow et al., 2016). However, a number of people with digestion problems such as small intestinal bowel infection or a colonic infection are not able to metabolize and absorb the nutrients in sweet potato (Gibson, Varney, Malakar, & Muir, 2015). Therefore, the pre-digestion is a desirable processing method which would improve bioavailability of nutrients and exert new bioactive compounds derived from the sweet potato substrates during fermentation (Duangjitcharoen et al., 2008).

Lactobacillus is one of the normal inhabitants in the human gastrointestinal (GI) tract and the dominant probiotic floras in the colon (Ren et al., 2014). It plays an important role in inducing host cells to generate antimicrobial peptides, improving gastrointestinal barrier function and competing with pathogens for epithelial adherence (Morrow, Gogineni, & Malesker, 2012). Currently, *Lactobacillus* was mainly used in dairy products for health beneficial effects including the prevention of diarrhea, stimulation of immune system and anti-inflammation of intestinal disorders (Ren et al., 2014). In fact, *Lactobacillus* could also convert some carbohydrates to organic acids and exhibit cinnamoyl esterase and decarboxylase activities for releasing phenolic compounds by taking advantages of food matrix (Barthelmebs, Diviés, & Cavin, 2001). As an economic plant, sweet potato is an ideal substrate to provide necessary nutrients to

support the growth of *Lactobacillus* which, in turn, has the potential to release conjugated bioactive compounds and produce secondary metabolites with more beneficial nutrition values. In order to evaluate the nutrition value of the fermented sweet potato, the change of phenolics, fatty acids, carotenoids and phytosterols in sweet potato after fermentation and the effect of different extract fractions on inhibiting pheochromocytoma derived cancer cell (PC-12) and normal monkey kidney cell (CV-1) proliferation were determined in this study. Therefore, the fermented sweet potato by efficient pre-digesting with *Lactobacillus* would be an economic and promising functional food product.

5.2 Materials and Methods

5.2.1 Chemicals and materials

HPLC grade acetonitrile, acetic acid and hexane were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Acetone and ethyl acetate were purchased from Macron (Charlotte, NC, USA) and EM Science (Gibbstown, NJ, USA), respectively. Standards of phenolic acids, fatty acids, phytosterols, carotenoids and cholesterol as well as the derivatization reagents trimethylsilyl imidazole (TMSIM) and BCl₃-methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Raw Garnet sweet potato was obtained from Whole Food Market in Baton Rouge, LA, US. Frozen cultures of *Lactobacillus acidophilus* LA-K (*Lb. acidophilus* LA-K) (Chr. Hansen's Laboratory, Milwaukee, WI) were stored at -35°C before use. The pheochromocytoma derived cancer cell (PC-12) and normal monkey kidney cell (CV-1) cell lines were purchased

from ATCC® (Manassas, VA, USA). Fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), Cell Titer Blue and antibiotic (penicillin–streptomycin) were ordered from Invitrogen (Grand Island, NY, US).

5.2.2 Fermentation of sweet potato mash and microbiological analyses

Five Garnet sweet potatoes were cleaned, peeled and cut into 1 cm cubes and ground by a kitchen blender with sterilized water at the ratio of 2:1 (wt/vol). A series of 250 mL pre-autoclaved flasks each containing 30g of the sweet potato mash were used for different treatments. Three treatments including boiling, hydrolysis and fermentation were used in this study, while the raw sweet potato mash served as the control group. For the boiling group, samples were boiled for 15 min with magnetic stirrer stirring during heating on a hot plate (PC-351 Corning, Corning, NY, USA). For the hydrolysis group, 15 mL hydrochloric acid solution (pH 2) was mixed with sweet potato mash (30 g) in the flask and the mixture was incubated at 37 °C for 30 min. Triplications of each treatment were carried out. The fermentation of the sweet potato mash was described as follows: two milliliters of freshly thawed *Lb. acidophilus* LA-K culture was suspended in 20 mL of sterile phosphate buffer (10 mM, pH 7.0) to obtain a working solution of 10% (vol/vol). Three flasks each containing 30 g sweet potato mash were aseptically inoculated with *Lb. acidophilus* LA-K working solution at 10% (wt/vol) and incubated at 37 °C for 24 h. The moisture content of each treatment was recorded for further standardizing the concentrations of the bioactive compounds. The pH of the fermented sweet potato mash

was measured using a pH meter (Orion 3 star Benchtop; Thermo Orion, Beverly, MA, USA). The colonies (CFU/mL) of *Lb. acidophilus* LA-K were counted before and after incubation by the standard plate method with Lactobacilli MRS broth after a 24 h inoculation at 37°C.

5.2.3 Extraction and determination of hydrophilic phenolic compounds and lipophilic fatty acids, phytosterols and carotenoids in sweet potato

Twenty grams of the sweet potato mash prepared from different treatments (boiling, hydrolysis and fermentation) and control (raw mash) was extracted with 20 mL of methanol three times at 60°C for 20 min. The methanol layer was combined after being centrifuged and transferred to a clean tube which was then subjected to solvent evaporation by a vacuum centrifuge evaporator (Labconco, Kansas City, MO, USA). The obtained extracts were used for the determination of hydrophilic phenolic compounds in control (HR), boiled (HB), hydrolyzed (HH) and fermented sweet potato mash (HF), respectively. While the lipophilic fraction of control (LR), boiled (LB), hydrolyzed (LH) and fermented sweet potato mash (LF) used for fatty acid, carotenoids and phytosterol determination was extracted with a mixture of hexane and ethyl acetate (50:50; v:v) and followed the same procedure as hydrophilic extraction. A working solution (10 mg/mL) of each hydrophilic and lipophilic extract was prepared by being re-dissolved with methanol and hexane, respectively.

For the cellular study, the hydrophilic and lipophilic extracts from raw and fermented sweet potato mash were extracted with the same procedure as described above but with a larger amount. While, in order to avoid the influence of carbohydrates on cell growth, the sweet potato mash samples (20 g) were purified by being extracted with acetone (20 mL) three times and the supernatant was collected and combined after centrifugation. Then, 50 mL of ethyl acetate was added to the solution and the upper layer was collected after completely mixed with a vortex. The ethyl acetate extract of raw (PHR) and fermented sweet potato mash (PHF) was further dried and weighed. All types of the extracts were stored in a -80 °C before use.

Phenolics Phenolic profile of Garnet sweet potato was determined by a HPLC (2690, Waters, Torrance, USA) and a diode array detector. Separations were carried out using a reversed phase column (C18, id 250×4.60 mm, 5 µm, Phenomenex, Torrance, USA) based on the method of Du et al. (2014). The hydrophilic extract (2 mL) was filtered by 0.45 µm microporous film before injection. The concentration of each compound was calculated based on their standards, while the caffeic acid derivative was obtained by using caffeic acid standard.

Fatty Acid The lipophilic extract (2 mL) was mixed with 200 µg of internal standard (C17:0) and 2 mL of BCl₃-methanol. After incubation at 60°C for 30 min, 1 mL of hexane was added to the reaction solution and vortexed. The upper hexane layer was collected and transferred to a clean test tube with 0.1 g anhydrous NaSO₄ at the bottom to

remove the potential moisture and transferred to a GC vial. GC (hp 5890, Hewlett-Packard, Wilmington, DE, USA) equipped with FID detector and Supelco SP2380 (30 m \times 0.25 mm) column (Bellefonte, PA, USA) was employed to determine the fatty acid profile. The GC program was the same as described in the study of Yue et al. (2008).

Phytosterol The determination of phytosterols was based on the study of Xu and Godber (1999) with slight modification. Two milliliter of the diluted lipophilic extract was transferred to a clean test tube and dried again. Then, 200 μ L of TMS and 50 μ L of acetonitrile were mixed with the extract at 65 $^{\circ}$ C for 30 min for derivatization. After extraction with 200 μ L of hexane, 2 μ L of the solution was injected. A Varian CP-3800 GC (Valnut Creek, CA, USA) interfaced with a Saturn 2200 mass spectrometer and a DB-5 column (L60 m \times i.d. 0.25 mm and df 0.25 μ m thin coating film) (Supelco, Bellefonte, PA, USA) was used for analysis. The oven temperature increased from 200 $^{\circ}$ C to 280 $^{\circ}$ C at the rate of 10 $^{\circ}$ C /min and was held at the final temperature for 62 min. Helium was the carrier gas at a constant flow rate of 1.5 mL/min with a split mode (1: 50). The injection port temperature was set at 280 $^{\circ}$ C.

5.2.4 The effect of raw and fermented sweet potato extracts on pheochromocytoma derived cancer cell (PC-12) and normal monkey kidney cell (CV-1) proliferation

The PC-12 cell line derived from a pheochromocytoma of the rat adrenal medulla and normal monkey kidney cell CV-1 cell line were used for the antiproliferative study. The cells were maintained in a CO₂ incubator with 5% CO₂ and 95% humidity and

supplemented with Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) and 1 % antibiotic (penicillin–streptomycin). The PC-12 cells or CV-1 were plated in a 96-well plate at a density of 3.0×10^3 cells/well after incubation for 48 h. The dried extracts of raw sweet potato (HR, LR, and PHR) and fermented sweet potato (HF, LF, and PHF) were dissolved with 0.2 % DMSO in PBS culture media as the test sample. Then, the PC-12 cell or CV-1 was exposed to a series of concentrations of sweet potato mash extracts (0.5, 0.2, 0.1, 0.05, and 0.02 mg/mL for PHR, PHF, LR and LF; 100.0, 50.0, 20.0, 10.0 and 5.0 mg/mL for HR and HF) and then incubated for 24 h at 37 °C. The cells mixed with only medium and DMSO were evaluated as blank. After incubation, the media was discarded and the cells were stained with 100 µL fresh media containing 20% Cell Titer Blue. The fluorescence intensity indicating the cell viability was obtained by a FluoStar Optima micro-plate reader (BMG, Germany) at excitation/emission wavelengths of 570/615 nm and reflected the number of survived cells. The percentage of survived cells was inversely proportional to the cell proliferation inhibition ability of different sweet potato extracts.

5.5 Data analysis

The determination of bioactive components of phenolics, fatty acids, phytosterols and carotenoids in the extracts was in triplicate and expressed as means \pm standard deviation by using Microsoft Excel (Redmond, WA). The significant differences among treatments were conducted by one-way ANOVA at $P < 0.05$ (SAS, 9.1.3, Cary, NY, US). The

results from cellular study were obtained from five repetitions for each treatment and analyzed by GraphPad Prism (Version 6.0, GraphPad Software Inc., USA). Results were considered statistically significant at $p < 0.05$ between treatments or concentrations by two-way ANOVA.

5.3 Results and Discussions

5.3.1 Fermentation characteristics of the sweet potato mash

As shown in Table 5.1, the pH dropped from 6.20 to 3.45 in the sweet potato mash after fermentation. This was due to the degradation of granular starch and the conversion of glucose via enzymatic pathway of *Lb. acidophilus* LA-K (Masiero, Peretti, Trierweiler & Trierweiler, 2014). Then, the bacteria would take advantage of glucose to synthesize lactic or acetic acids and release CO₂ which were responsible for lowering the pH of the sweet potato mash (Cui, Liu, Li, & Song, 2011). On the other hand, the sweet potato had protein and carbohydrates which are important elements for providing sufficient source of nitrogen and carbon for the bacteria. As the results showed, the viable cell counts of *Lb. acidophilus* LA-K in the fermented sweet potato increased from $3.80 \pm 0.01 \times 10^6$ to $7.48 \pm 0.67 \times 10^8$ CFU/mL after a 24 h incubation at 37°C. Thus, it indicated that the sweet potato mash is a great substrate for the growth and biotransformation performance of *Lb. acidophilus* LA-K.

Table 5.1 Change of pH and visible cell of *Lactobacillus acidophilus* LA-K in Garnet sweet potato after fermentation

Compounds	0h	24h
pH Value	$6.20 \pm 0.15a$	$3.45 \pm 0.02b$
Viable cell (cfu/mL)	$3.80 \pm 0.01 \times 10^6 a$	$7.48 \pm 0.67 \times 10^8 b$

Mean values with the different letters are significantly different ($p < 0.05$)

5.3.2 The changes of hydrophilic phenolic acids in sweet potato after fermentation

Generally, the phenolic acids found in sweet potato are mainly chlorogenic acid and its related derivatives. Raw Garnet sweet potato contained five phenolic acids including chlorogenic, caffeic, 4,5-dicaffeoylquinic, 3,5-dicaffeoylquinic and 3,4-dicaffeoylquinic acids. Their chromatograms are shown in Figure 5.1 and the concentrations listed in Table 5.2 showed that 3,4-dicaffeoylquinic acid (29.12 ± 1.68 mg/100g DW) was the dominant phenolic acid, while the other phenolic acid was in a range of 2.39 mg/100g DW for caffeic acid to 5.00 mg/100g DW for 4,5-dicaffeoylquinic acid.

With the involvement of fiber, starch, carbohydrate or protein, most phytochemicals in sweet potato are trapped in the cores of starch and protein, or in bounded and conjugated forms with low bioavailability (Khoddami, Wilkes, & Roberts, 2013). In order to release and absorb those health-benefiting compounds, further degradation or digestion are needed which would cause extra burdens for gastrointestinal system in human body, especially for those who suffer from small bowel infection or colonic infection (Gibson, Varney, Malakar, & Muir, 2015). Generally, boiling is

believed to disrupt the amylose-amylopectin structure of the starch complex, which could release some trapped phytochemicals (Bahado-Singh et al., 2011). As shown in Table 5.2, a new caffeic acid derivative appeared and the other phenolic acids were significantly higher than those in raw sweet potato except 3,4-dicaffeoylquinic acid. The increase of the phenolic content in boiled samples could be attributed to the release of bound phenolics and inactivation of polyphenoloxidase by the heat treatment (Truong et al., 2007).

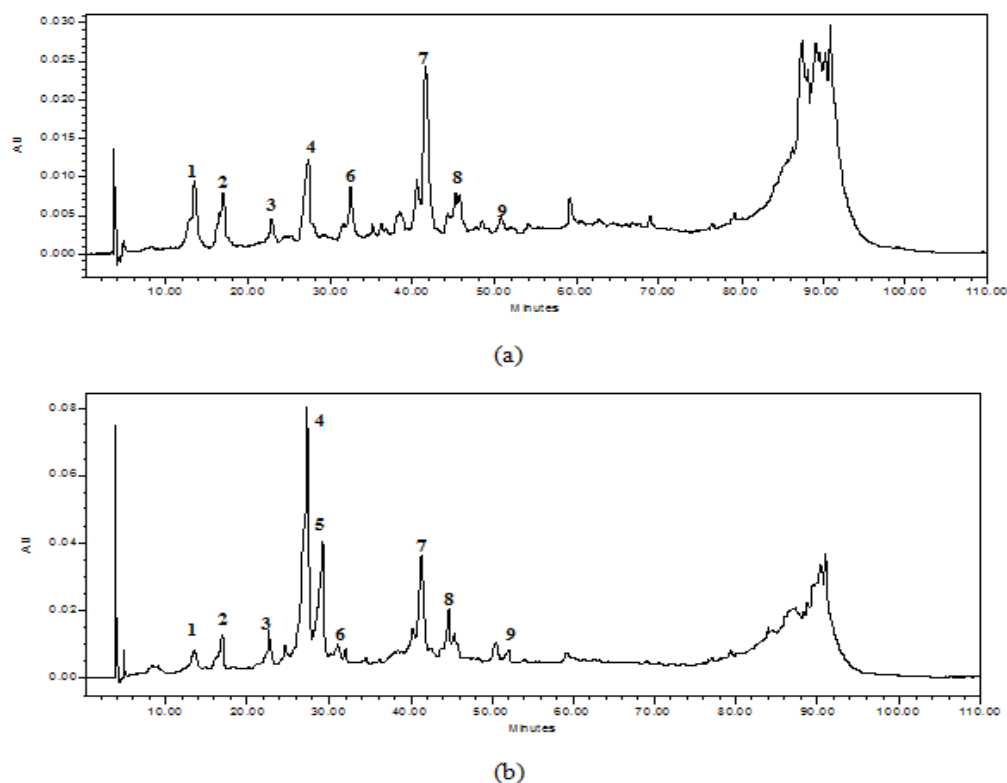


Figure 5.1 Typical chromatograms of hydrolyzed (a) and fermented (b) sweet potato mash 1) chlorogenic acid; 2) caffeic acid; 3) caffeic acid derivative; 4) *p*-coumaric acid; 5) ferulic acid; 6) 4,5-dicaffeoylquinic acid; 7) 3,5-dicaffeoylquinic acid; 8) 3,4-dicaffeoylquinic acid; 9) cinnamic acid

Table 5.2 Phenolic profiles of raw, boiled, hydrolyzed and fermented sweet potato (n=3)

	Phenolics (mg/100g DW)	Raw Sweet Potato	Boiled Sweet Potato	Hydrolyzed Sweet Potato	Fermented Sweet Potato
1	Chlorogenic acid	4.26 ± 0.05 <i>bD</i>	7.05 ± 0.26 <i>cB</i>	16.85 ± 0.04 <i>bA</i>	5.74 ± 1.25 <i>eC</i>
2	Caffeic acid	2.39 ± 0.02 <i>dC</i>	3.16 ± 0.55 <i>eB</i>	3.77 ± 0.66 <i>dB</i>	10.22 ± 0.34 <i>dA</i>
3	Caffeic acid derivative	N.D.	0.98 ± 0.02 <i>fC</i>	1.78 ± 0.25 <i>eB</i>	4.77 ± 1.21 <i>eA</i>
4	<i>p</i> -Coumaric acid	N.D.	N.D.	3.52 ± 0.10 <i>dB</i>	26.42 ± 2.01 <i>bA</i>
5	Ferulic acid	N.D.	N.D.	N.D.	19.10 ± 1.87 <i>c</i>
6	4,5- Dicaffeoylquinic acid	5.00 ± 0.96 <i>bC</i>	8.29 ± 0.63 <i>bB</i>	10.95 ± 1.43 <i>cA</i>	3.17 ± 0.02 <i>fD</i>
7	3,5- Dicaffeoylquinic acid	3.14 ± 0.41 <i>cD</i>	6.08 ± 0.28 <i>dC</i>	11.36 ± 1.16 <i>cB</i>	23.34 ± 0.75 <i>bA</i>
8	3,4- Dicaffeoylquinic acid	29.12 ± 1.68 <i>aB</i>	21.13 ± 0.21 <i>aD</i>	23.34 ± 0.40 <i>aC</i>	31.90 ± 1.77 <i>aA</i>
9	Cinnamic acid	N.D.	N.D.	1.23 ± 0.18 <i>fB</i>	2.70 ± 0.51 <i>gA</i>
	<i>Total</i>	43.91	46.69	72.80	127.36

*N.D. not detected.

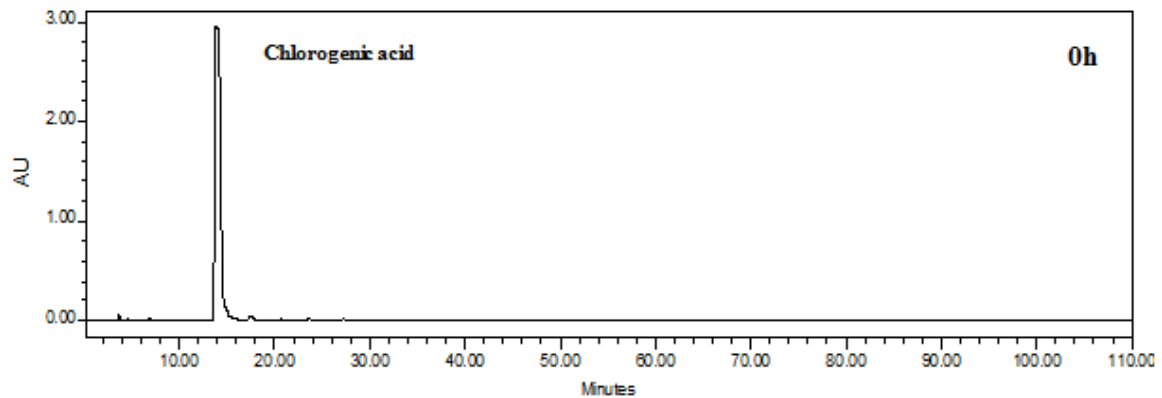
*Values with different lower case letters in a column and capital letters in a row are significantly different at $p < 0.05$.

Compared with raw sweet potato, three more phenolic compounds caffeic derivative, *p*-coumaric and cinnamic acid were found after acid hydrolysis. The concentration of chlorogenic acid (16.85 ± 0.04 mg/100g DW) in hydrolyzed samples was approximately twice and four times higher than that in raw and boiled sample, respectively. Additionally, hydrolysis helped release twice and four times of 4,5-dicaffeoylquinic and 3,5-dicaffeoylquinic acids than raw sweet potato and significantly higher than boiling treatment. The observed increased phenolic acids could be due to the acid solution that assisted the release of phenolic molecules which have ester and ether linked with cell wall polymers and binding to fibers (Xu, 2012).

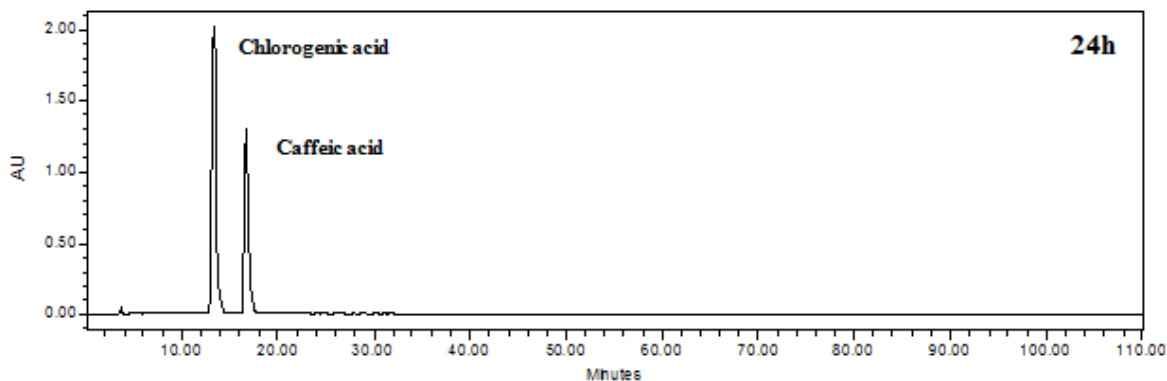
Though boiling and hydrolysis were effective in releasing phenolic compounds from sweet potato mash, both types of processing methods easily cause degradation of other potential phytochemicals and would be the major limitations applied in food system. However, the bio-processing method such as fermentation, is able to improve food inherent functional value and bioavailability in human body with the bioconversion of microorganisms. As a common probiotic in intestine, *Lactobacillus acidophilus* has been evidenced to help balancing gut microbiota, inhibiting pathogenic infection, lowering blood cholesterol and reducing the risks of colon cancer (Huang et al., 2014). In this study, after fermentation with *Lb. acidophilus* LA-K, the concentrations of most phenolic acids in Garnet sweet potato significantly increased (Table 5.2). For example, the caffeic acid (10.22 ± 0.34 g/100g DW) in the fermented sample was about four times of that in raw sample (2.39 ± 0.02 g/100g DW) and three times higher than boiled (3.16 ± 0.55 g/100g DW) or hydrolyzed sample (3.77 ± 0.66 g/100g DW) (Table 5.2). However, compared with boiled (7.05 ± 0.20 g/100g DW) and hydrolyzed samples (16.85 ± 0.04 g/100g DW), chlorogenic acid decreased to 5.74 ± 1.25 g/100g DW in fermented sample which corresponded to the accumulation of caffeic acid in approximately equivalent concentration. In order to prove the relationship of chlorogenic acid and caffeic acid, the chlorogenic acid standard was incubated with *Lb. acidophilus* LA-K at MSR media for 24 h and the result demonstrated that *Lb. acidophilus* LA-K was responsible for the biotransformation of chlorogenic acid to caffeic acid (Figure 5.2). Similar result has been reported in the study of Bel-Rhlid et al., (2013), in which, *L. johnsonii* NCC 533 could convert chlorogenic acid to caffeic acid in green coffee extract. The biotransformation might be due to *Lb.* species that contain chlorogenate esterase and hydroxycinnamate

decarboxylase which exhibited high affinity and catalytic efficiency toward aromatic compounds such as chlorogenic acids (Bel-Rhliid et al., 2013). Generally, ferulic and *p*-coumaric acid are hydroxycinnamic acids and most of them are in bound form as ester-linked in the cell wall polysaccharides, organic acids, lipids or protein of plants (Szwajgier, 2010). Among the four treatments, hydrolyzed and fermented samples contained *p*-coumaric acid at levels of 3.52 ± 0.10 and 26.42 ± 2.01 mg/100g DW, respectively, while the cinnamic acid (mg/100g DW) was 1.23 ± 0.18 for hydrolyzed and 2.70 ± 0.51 for fermented samples, respectively (Table 5.2). However, they were not detected in raw and boiled samples (Table 5.2). Ferulic acid was only found in the fermented sample with a concentration of 19.10 ± 1.87 mg/100g DW (Table 5.2). It has been reported that *Lactobacillus* species secretes active extracellular cellulolytic enzyme such as feruloyl esterases, thus, it could de-esterify dietary fiber and release free ferulic, *p*-coumaric and cinnamic acid from plant cell wall (Esteban-Torres et al., 2013). Also, the presence of ferulic acid and increase of *p*-coumaric acid in fermented sweet potato mash could result from their ferulate form via biotransformation of *Lb. acidophilus* LA-K during fermentation (Esteban-Torres et al., 2013). In the study of Snook et al. (1994), hexadecyl, octadecyl, and eicosyl *p*-coumarates as well as hexadecyl (Z)- and (E)-ferulates were determined in sweet potato root latex, and they were potential substrates for *Lb. acidophilus* LA-K to generate free ferulic acid or *p*-coumaric acid from methyl ferulate or methyl *p*-coumarate (Szwajgier, 2010). Numerous clinic studies have demonstrated free caffeic acid could be more easily absorbed in the stomach and small intestine than chlorogenic acid (Olthof et al., 2001). Also, ferulic acid and *p*-coumaric could easily get access to blood plasma (Olthof et al., 2001). Therefore, the releasing of

higher level of phenolic compounds, especially caffeic, ferulic and *p*-coumaric acids would enhance bioactive performance of sweet potato after fermentation.



(a)



(b)

Figure 5.2 The chromatograms of chlorogenic acid standard fermented *Lb. acidophilus* LA-K at 0h (a) and 24h (b)

5.3.3 The changes of lipophilic fatty acids, phytosterols and carotenoids in sweet potato after fermentation

The fatty acid composition of the sweet potato with different treatments was investigated (Table 5.3). In raw sweet potato, the most abundant fatty acid was linoleic acid (C18:2n-6) (85.67 ± 5.56 mg/100g DW), followed by palmitic acid (C16:0), myristic acid (C14:0), linolenic acid (C18:3n-3) and stearic acid (C18:0) ranging from 47.18 ± 3.24 to 17.96 ± 0.61 mg/100g DW.

Table 5.3 Fatty acids, phytosterols and carotenoids in raw, boiled, hydrolyzed and fermented sweet potato (n=3)

(mg/100g DW)	Compounds	Raw Sweet Potato	Boiled Sweet Potato	Hydrolyzed Sweet Potato	Fermented Sweet Potato
Fatty Acid	Myristic acid (C14:0)	44.64 ± 3.67 bB	33.62 ± 2.55 aC	22.24 ± 2.26 cD	80.61 ± 5.96 cA
	Palmitic acid (C16:0)	47.18 ± 3.24 bC	16.67 ± 0.62 bD	54.78 ± 3.17 bB	109.29 ± 3.74 bA
	Stearic acid (C18:0)	17.96 ± 0.61 dB	17.27 ± 0.24 bB	12.50 ± 1.17 dC	42.86 ± 0.87 eA
	Linoleic acid (C18:2n-6)	85.67 ± 5.56 aC	30.98 ± 2.94 aD	107.31 ± 6.34 aB	171.07 ± 12.77 aA
	Linolenic acid (C18:3n-3)	22.08 ± 2.66 cC	3.45 ± 0.49 cD	24.10 ± 1.46 cC	66.05 ± 2.24 dA
	<i>Total</i>	217.53	101.99	220.93	469.88
Phytosterol	Campesterol	3.38 ± 0.19 bB	2.98 ± 0.22 bB	3.07 ± 0.12 bB	4.02 ± 0.15 bA
	Stigmasterol	0.74 ± 0.04 cB	0.70 ± 0.03 cB	0.85 ± 0.63 cA	0.86 ± 0.03 cA
	β-Sitosterol	9.94 ± 0.58 aC	8.27 ± 0.59 aD	10.86 ± 0.02 aB	12.20 ± 0.85 aA
	<i>Total</i>	14.06	11.95	14.78	17.08
Carotenoids	Lycopene	17.60 ± 0.39 cB	2.49 ± 0.17 cD	20.40 ± 2.14 cA	12.72 ± 0.72 cC
	Lutein	32.51 ± 2.55 bA	8.58 ± 1.66 bC	34.66 ± 3.17 bA	24.58 ± 3.17 bB
	β-carotene	438.64 ± 39.86 aA	188.09 ± 3.97 aC	445.85 ± 13.25 aA	318.82 ± 13.50 aB
	<i>Total</i>	488.75	199.16	500.91	356.12

*Values with different lower case letters in a column and capital letters in a row are significantly different at $p < 0.05$.

However, the level of each fatty acid significantly decreased in boiled sweet potato, especially linoleic and linolenic acid with a high degree of unsaturation. Similar results were reported in the study of Longo et al. (2012) that heat treatment (60-100°C) resulted in degradation of unsaturated fatty acids in tomatoes which was due to oxidation catalyzed by heat. In addition to free forms, the fatty acids also bind with phytosterols, glycerols, phospholipids and sugar-containing glycolipids in plants as important membrane constituents in the chloroplasts and mitochondria (Baxter, Harborne, & Moss, 1998). In this study, the hydrolyzed sweet potato had higher levels of palmitic, linoleic and linolenic acids and a slight increasing amount of the stigmasterol and β-sitosterol

compared with the raw sweet potato (Table 5.3). Therefore, the increment might be obtained from release of free fatty acids trapped in the starch granules or the breakdown of bindings from phytosterol esters by acid hydrolysis. After fermented with *Lb. acidophilus* LA-K, the linolenic acid (171.07 ± 12.77 mg/100g DW) was approximately three times higher and the other determined fatty acids (ranged from 42.86 ± 0.87 to 109.29 ± 3.74 mg/100g DW) were approximately twice higher than each of them in raw sweet potato. The increase of the fatty acids might result from enzymatic action based de-esterification of bound fatty acids and the contribution of *Lb. acidophilus* LA-K which also produce palmitic, myristic and stearic acid during growth (Johnsson et al., 1995). Since the fatty acids, especially unsaturated ones help support brain cells growth, neurological development and cognitive function, the accumulation of the fatty acid would enhance the nutritional value of the fermented sweet potato (Panickar & Bhathena, 2010). Also, raw, boiled and hydrolyzed sweet potato had little difference in campesterol, stigmasterol and β -sitosterol contents, while the three phytosterols exhibited increasing levels after fermentation (Table 5.3). Thus, they would help improve the nutrition value of fermented sweet potato as the phytosterols were recommended for consumption to inhibit intestinal cholesterol absorptions and to control cholesterol concentrations in plasma due to the similar structure of cholesterol but with different side chains (Ishida, 2014). Among the three determined carotenoids, β -carotene was the dominant (438.64 ± 39.86 mg/100g DW) which was followed by lutein (32.51 ± 2.55 mg/100g DW) and

lycopene (17.60 ± 0.39 mg/100g DW) in raw sweet potato, while each of the carotenoids slightly increase in the hydrolyzed samples. However, different degree of reduction of carotenoids profile was observed in boiling and fermentation treatments which were due to the long time heat or light exposure (Table 5.3).

On the other hand, the *Lactobacillus acidophilus* is a probiotic bacteria species which could help reducing overgrowth of pathogens, stimulating an immune response, improving the blood lipid metabolism or potentially killing cancer cells in human gastrointestinal tract (Sanders & Klaenhammer, 2001). Therefore, the fermented sweet potato would be considered an ideal food system to enhance the bioavailability of the bioactive compounds of sweet potato, but also deliver *Lactobacillus acidophilus* for its probiotic performance. As a result, the hydrophilic and lipophilic raw and fermented sweet potato extracts were selected and compared in the following cellular study.

5.3.4 The effect of raw and fermented sweet potato extracts on pheochromocytoma derived cell (PC-12) and normal monkey kidney cell (CV-1) proliferation

The anticancer potentials of hydrophilic, purified hydrophilic and lipophilic extracts of raw sweet potato versus fermented sweet potato were evaluated by the inhibition efficiency on cancer cell PC-12 growth. Figure 5.3a demonstrated that purified hydrophilic extracts of raw (PHR) and fermented (PHF) sweet potato caused dose-dependent inhibition of PC-12 cell proliferation after 24 h incubation. As the extract concentration increased to 0.2 mg/mL, the viable cancer cells dropped to 9.5% in PHF,

while there was 63.2% remained in PHR (Figure 5.3a). The highest inhibitory rate was achieved at a concentration of 0.5 mg/mL, at which level, only 30.1% and 2.1 % of the cells survived in purified hydrophilic extracts of raw (PHR) and fermented (PHF) sweet potato, respectively (Figure 5.3a). Thus, the greater performance of PHF was due to the higher levels and abundant free-form phenolic compounds than those of PHR. For example, there were four more phenolics including caffeic acid derivative, *p*-coumaric, ferulic and cinnamic acids in fermented sweet potato than raw sweet potato (Table 5.2). Also, compared with raw sweet potato, the concentration of caffeic acid or 3,5-dicaffeoylquinic acid in fermented sweet potato was approximately four and seven times higher (Table 5.2). Similar result was found in the study of Rocha et al. (2012), who reported that caffeic acid, 3,4-, 3,5- and 4,5- dicaffeoylquinic acid as well as ferulic and *p*-coumaric acid could inhibit colon cancer cell lines including RKO, HT-29 and Caco-2.

There was no significant difference between lipophilic extract of raw (LR) and fermented (LF) sweet potato in inhibiting PC-12 cell growth below 0.1 mg/mL (Figure 5.3b). As the extract concentration increased to 0.2 mg/mL, the inhibition efficiency of LF was significantly higher than LR (Figure 5.3b). However, with the same concentration of 0.5 mg/mL, LF and LR contained 44.6 and 53.4 % of viable cancer cells which was much higher than that of PHF (2.1%) and PHR (30.1%) (Figure 5.3a & 5.3b).

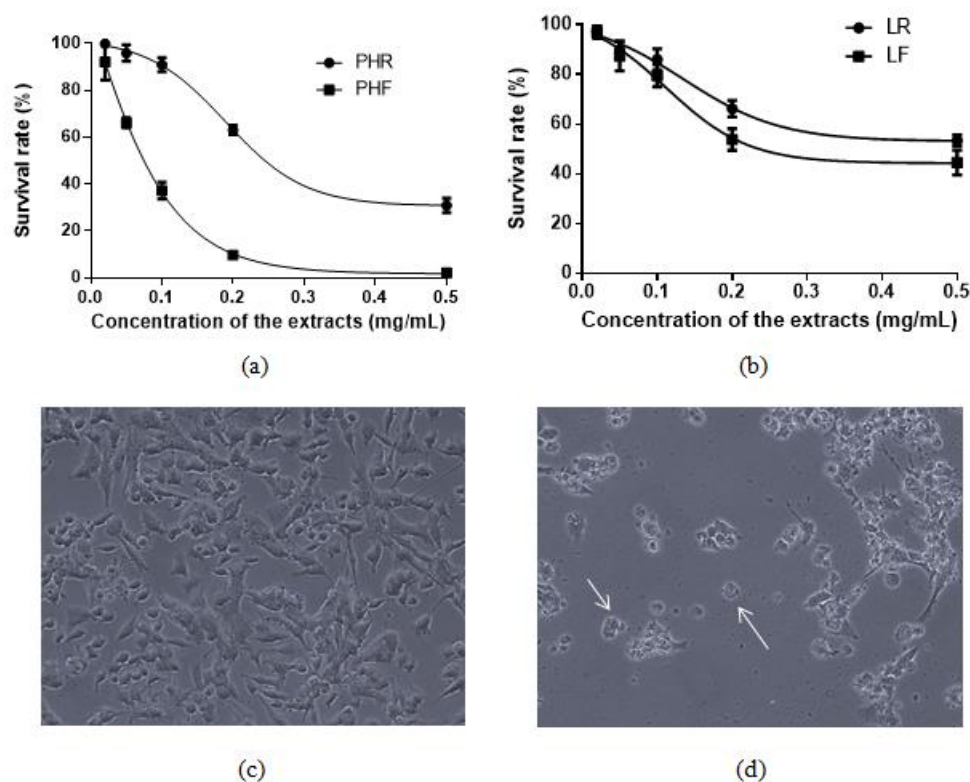


Figure 5.3 The inhibition of PC-12 cell proliferation by (a) purified hydrophilic raw (PHR) and fermented sweet potato (PHF); (b) lipophilic extracts of raw (LR) and fermented sweet potato (LF); and the morphology of normal PC-12 cell (c) and apoptotic PC-12 cell (d)

As reported in the study of Upadhyay et al. (2007), carotenoids could deregulate the cancer cell proliferation rate by interfering different phases of the cell cycle, therefore, it was possible that the abundant carotenoids (488.75 mg/100g DW for LR and 356.12 mg/100g DW for LF) in the lipophilic extracts that mainly contributed to the capability in inhibiting the cancer cell proliferation. Although phytosterols also play an important role in suppressing the growth of the cancer cell, its total content was only 14.06 and 17.08 mg/100g DW in LR and LF, respectively, which may have limited its anti-cancer efficiency (Table 5.3). In addition, the fatty acids concentrations rose dramatically in

fermented sweet potato, however, they were not the primary factors that were responsible for the anticancer performance of the lipophilic extracts. Thus, the LR and LF exhibited relatively weaker anti-cancer potential than PHR and PHF.

The interaction of lipophilic and hydrophilic bioactive compounds with the cancer cell is based on different accessibility and inhibition pathway. Generally, lipophilic compounds could easily pass across the lipid bilayers of biomembranes by simple diffusion or special membrane proteins for transferring such no-polar solutes (Alberts et al., 2002). Distinctive from that of lipophilic substances, most hydrophilic compounds have difficulties in penetrating the cell membrane. However, at physiologic pH, the multiple hydroxyl groups in small molecule phenolics could interact with the polar head groups of phospholipids at the membrane surface via the formation of hydrogen bonds (Manach et al., 2004). It would then increase pH and the penetration of the phenolics would be improved by deprotonation of the hydroxyl groups and variations in membrane structure and fluidity (Castelli et al., 1999). Compared with the control cancer cell shown in Figure 5.3c, the speeded up apoptotic progress of cancer cells was induced by lipophilic or purified hydrophilic sweet potato extracts which resulted in the presence of cell shrinkage, apoptotic bodies, and margination of the nucleus indicated by arrows (Figure 5.3d). However, the anti-cancer potential of the hydrophilic phytochemicals would be affected since hydrophilic extracts of sweet potato contained a large amount of

carbohydrates but much fewer equivalents of phenolic compounds as purified hydrophilic extracts. Thus, it would have side influence on cancer cell growth.

As shown in Figure 5.4b, both HR and HF helped to remain the growth of the cancer cell at a relatively low concentration (<1 mg/mL). Similar result was demonstrated in the study of Masur et al. (2011) that with progressive tumorigenesis, the promotion of cell proliferation, progression of cell cycle, anti-apoptotic signaling and angiogenesis in tumor cell lines from several organs were observed at a diabetogenic glucose concentration (11 mM) compared to physiological one (5.5 mM). At the same concentration of 0.5 mg/mL with lipophilic (LR and LF) and purified hydrophilic extracts (PHR and PHF), HR and HF exhibited much weaker inhibitory efficiency (Figure 5.4a). However, the viability of the cancer cell rapidly reduced to 21.11 % in HF and 35.42 % in HR as their concentration increased to 50 mg/mL and finally exhibited 100% inhibitory efficiency in both HR and HF at the concentrations as high as 100 mg/mL (Figure 5.4a). The significant reduction of the survival rate at high concentrations could be explained by osmotic pressure of the high carbohydrate concentration which caused the aggregation and morphological variation of the cells as marked with arrow in Figure 5.4b.

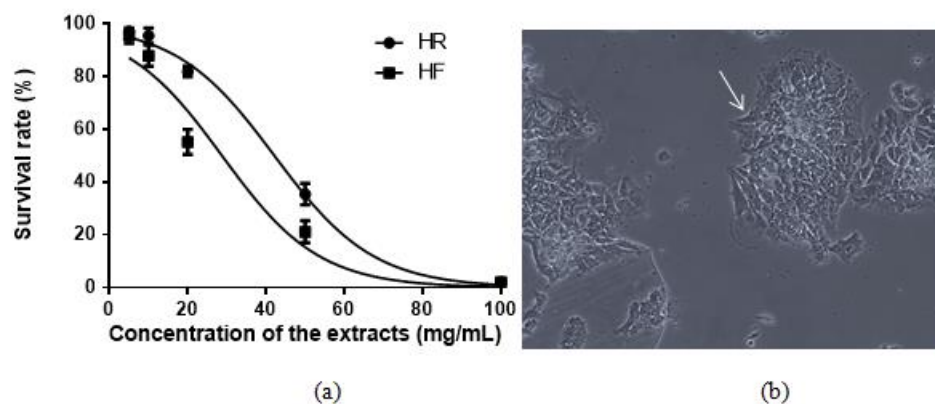


Figure 5.4 The inhibition of PC-12 cell proliferation by hydrophilic extracts of fresh (HR) and fermented sweet potato (HF) (a) and the morphology of dead PC-12 cell (b)

On the other hand, the priority of the applying plant-derived bioactive extracts as alternative cancer remedies is to effectively reduce the incidence of cancers but with low toxicities and low side effects compared with other cancer treatments such as chemotherapeutic agents or radiation. In this study, the monkey kidney cell lines (CV-1) were used for assessing the influence of different sweet potato extracts on the growth of normal cells. Figure 5.5 elucidated that little inhibition effect was observed in CV-1 cells treated with the same concentrations of the extract as in PC-12 cell lines. For example, relatively low concentration (0.02 mg/mL) of PHR and PHF slightly assisted the CV-1 growth. Even at the maximum concentration of 0.5 mg/mL, only 22.37-24.74% or 16.75-19.75% of CV-1 cells were inhibited in purified hydrophilic extracts and lipophilic extracts, respectively (Figure 5.5).

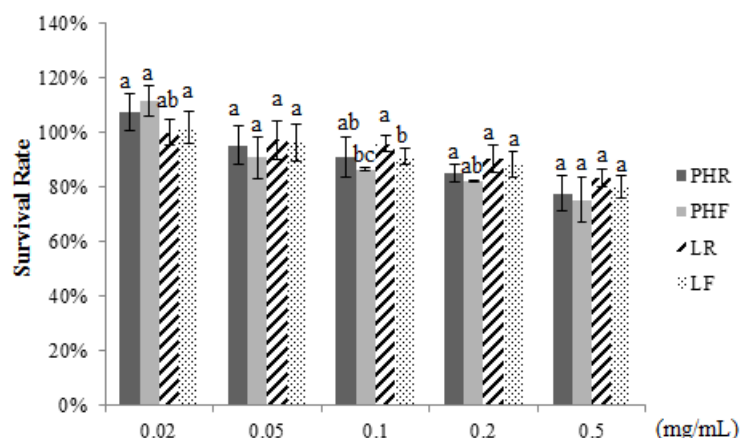


Figure 5.5 The effect of PHR, PHF, LR and LF on CV-1 cell proliferation
Bars with different letters are significantly different ($p < 0.05$)

The high inhibitory efficiency of the plant derived extracts on cancer cell but weak influence on normal cells could be explained by their different energy metabolisms. Generally, normal cells with competent mitochondria undergo Krebs Cycle metabolic pathway and generate the majority of ATP for cell growth (Wen, Zhu, & Huang, 2013). However, most cancer cells have dysfunction in mitochondrial, and rely more on the glycolytic pathway in the cytosol to generate the metabolic intermediates and ATP (Wen, Zhu, & Huang, 2013). Thus, the key enzymes in glycolytic pathway have been considered as potential therapeutic targets for inhibition of cancer cell proliferation. Due to the non-covalent interactions with proteins, phenolic compounds have been reported as the inhibitors of key enzymes in glycolytic pathway, such as glucosidases, xanthine oxidase and PKM2 activities which further perturb proliferation of the cancer cell (Aslan, Guler, & Adem, 2015). As for the lipophilic phytosterols and carotenoids, they could

interrupt balance between cancer cell proliferation and apoptosis by exerting interference with protein phosphatase 2A (PP2A) in sphingomyelin cycle and block the cell cycle at G0/G1 phase in prostate cancer, hepatocyte, and breast cancer cell lines (Awad & Fink, 2000; van Breemen & Pajkovic, 2008). Therefore, phytochemicals could be used as either mono-treatments or in association with classical chemotherapeutic drugs, increasing the therapeutic efficacy on cancer cells and lowering the toxic side effects on normal cells.

5.4 Conclusion

In summary, this study demonstrated that fermentation of Garnet sweet potato with probiotic *Lb. acidophilus* LA-K could significantly increase the content of most hydrophilic phenolic acids, and lipophilic fatty acids or phytosterols via enzymatic action based bio-releasing or bioconversion of the microorganisms. However, it slightly reduced the carotenoids level. Also, the fermented sweet potato extracts with a higher level and abundant phytochemicals exhibited higher efficiency than raw extracts in inhibiting the pheochromocytoma derived cancer cell (PC-12) proliferation. The purified hydrophilic extracts of raw or fermented extracts had greater anticancer potential than their corresponding lipophilic extracts. However, there were minimal effects on the normal monkey kidney cell (CV-1) growth because those phytochemicals targeted on deactivating the key enzymes of glycolytic pathway in cancer cells energy metabolism

but has less influence on the Krebs Cycle pathway for normal cells. Therefore, fermentation with *Lb. acidophilus* LA-K is a valuable biotechnological approach for sweet potato nutritional value enrichment, and bioavailability improvement, and the fermented extracts could be further applied as promising therapeutic ingredients for cancer treatment.

5.5 References

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CHAPTER 6 SUMMARY AND CONCLUSIONS

Chapter 2 reported the levels and profiles of hydrophilic and lipophilic antioxidant phytochemicals in sweet sorghum millets. The dominant hydrophilic and lipophilic antioxidants were apigeninidin and γ -tocopherol, respectively. The hydrophilic antioxidants showed higher antioxidant activity than lipophilic antioxidants in both the DPPH assay and the cholesterol-linoleic acid oxidation model. Therefore, the health benefits of sweet sorghum may be mainly attributed by its hydrophilic antioxidant phytochemicals. Thus, sweet sorghum (Dura) could be a valuable biomaterial for health promoting applications.

Chapter 3 investigated the phenolic profiles of Thai holy and sweet basil seeds and leaves. This is the first study to investigate the anti-lipid-oxidation capabilities of Thai holy and sweet basil seeds and leaves by using a cholesterol emulsion model. In this study, rosmarinic, caftaric, chlorogenic, p-hydroxybenzoic and protocatechuic acid were detected in both THBL and TSBL. Caffeic acid was only found in Thai holy basil. Additionally, THBS had seven primary phenolics including rosmarinic, caffeic, chlorogenic, p-hydroxybenzoic, p-coumaric, protocatechuic acid and rutin, while only p-hydroxybenzoic and rutin were found in TSBS. Thai holy basil leaves exhibited greater anti-lipid-oxidation performance than its seeds or Thai sweet basil leaves and seeds. Therefore, Thai holy basil leaves could be processed as an antioxidant ingredient to be applied in

various food products. In our future study, animal and cell system will be applied to verify the health promoting function of the Thai holy basil leaves.

Chapter 4 demonstrated the concentration and profile of phenolics, tocopherols, phytosterols and fatty acids in butterfly pea seeds and petals. HEp-2 carcinoma cell line was used to evaluate the anticancer capability of butterfly pea seeds and petals extracts (HBP, HBS, LBP and LBS). The results indicated HBS was the most efficient in inhibiting the viability of HEp-2 cells. Both HBS and HBP exhibited greater performance than LBS and LBP in inhibiting the cells proliferation. Therefore, the hydrophilic and lipophilic butterfly pea seeds and petals extracts possess health promoting function and could be used as a functional food ingredient or potential cancer therapy supplement.

Chapter 5 summarized that fermentation of Garnet sweet potato with probiotic *Lb. acidophilus* LA-K can significantly increase the content of most hydrophilic phenolic acids, and lipophilic fatty acids or phytosterols via enzymatic action based bio-releasing or bioconversion of the microorganisms. However, it slightly reduced the carotenoids level. Also, the fermented sweet potato extracts with a higher level and abundant phytochemicals exhibited higher efficiency than raw extracts in inhibiting the pheochromocytoma derived cancer cell (PC-12) proliferation. The purified hydrophilic extracts of raw or fermented extracts had greater anticancer potential than their corresponding lipophilic extracts. However, these extracts had minimal impact on the

normal monkey kidney cell (CV-1) growth because they targeted on deactivating the key enzymes of glycolytic pathway in cancer cells energy metabolism but has less influence on the Krebs Cycle pathway for normal cells. Therefore, fermentation with *Lb. acidophilus* LA-K is a valuable biotechnological approach for sweet potato nutritional value enrichment, and bioavailability improvement, and the fermented extracts could be further applied as promising therapeutic ingredients for cancer treatment.

**APPENDIX A: THE LETTER OF PERMISSION OF PUBLISHED PAPER
“PHYTOCHEMICALS IN SWEET SORGHUM (DURA) AND THEIR ANTIOXIDANT
CAPABILITIES AGAINST LIPID OXIDATION”**



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APPENDIX B: THE LETTER OF PERMISSION OF PUBLISHED PAPER “COMPARISON OF PHENOLIC PROFILES AND ANTIOXIDANT POTENTIALS OF THE LEAVES AND SEEDS OF THAI HOLY AND SWEET BASILS”



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VITA

Yixiao Shen was born in May, 1987, Shenyang, China. In June 2010, she graduated from Shenyang Agricultural University with a Bachelor of Food Quality and Safety. After receiving her bachelor's degree, she was recommended for Master study without examination majoring in Food Engineering at Shenyang Agricultural University in September, 2010. She joined the Ph.D. program in the School of Nutrition and Food Sciences at Louisiana State University in 2011 under the mentorship of Dr. Zhimin Xu and Dr. Witoon Prinyawiwatkul. She will receive degree of Doctor of Philosophy in Food Science during the December 2015 commencement ceremony.